REMARKS

Applicants note that the Summary page of the December 17, 2002 Office Action indicates that the Office Action is "Final." However, Applicants' undersigned attorney telephoned Examiner Cook on January 20, 2003 to inquire if the Office Action was in fact intended to be made Final. Examiner Cook stated that the first page was marked incorrectly and that the December 17, 2002 Office Action is a non-Final Action.

Claims 1-3, 5, 8, 12, 13, and 18-26 were pending in the application. Claims 1-3, 12, 13, and 18-24 previously have been withdrawn from consideration. Applicants have amended claims 25 and 26 for clarity. The claim amendments do not introduce any new matter and thus, their entry is requested. Upon entry of the present Amendment, claims 5, 8, 25, and 26 will be currently pending and under examination in the application.

Objection to Drawings

The Examiner indicated that the Drawings remain objected to by the Draftsperson. The Examiner has further acknowledged that Applicant has deferred corrective action until allowance.

Objection to claims 5, 8, 25, and 26

The Examiner has objected to claims 5, 8, 25, and 26 because the dependent claims do not refer to a previous claim. The Examiner has indicated that this rejection will be maintained until correction after allowance, at which time the claims will be renumbered.

Rejection Under 35 U.S.C. §112, second paragraph

The Examiner rejected claims 5, 8, 25, and 26 under 35 U.S.C. §112, second paragraph, as allegedly being indefinite. Specifically, the Examiner has taken the position that the claims do not clearly identify what the claimed antibodies will bind.

In response, without conceding the correctness of the Examiner's position, but to advance prosecution of the subject application, Applicants have amended claims 25 and 26 to more clearly identify for the Examiner the binding characteristics of the claimed antibodies and antibody fragments. These amendments do not alter the scope of the claims, but merely represent grammatical clarifications. As such, the claims, as amended, continue to clearly identify the particular osteocalcin fragments to which the claimed antibodies and antibody fragments will bind, namely, osteocalcin fragments selected from the group consisting of i) a fragment which spans from amino acid in position 7 to amino acid in position 30 of the amino acid sequence set forth in SEQ ID NO:2 in which all three glutamic acids in positions 17, 21 and 24 of said sequence are gamma-carboxylated, and ii) a fragment which spans from amino acid in position 6 to amino acid in position 30 of the amino acid sequence of SEQ ID NO:2 in which all three glutamic acids in the positions 17, 21 and 24 of said sequence are gamma-carboxylated. Applicants maintain that the claims, as amended, particularly point out and distinctly claim the subject matter that the Applicants regard as the invention. Accordingly, Applicants respectfully request that the Examiner reconsider and withdraw the rejection of claims 5, 8, 25, and 26 under 35 U.S.C. §112, second paragraph.

Claim Rejections Under 35 U.S.C. §112, first paragraph

Written Description

The Examiner rejected claims 5, 8, 25, and 26 under 35 U.S.C. §112, first paragraph as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventors had possession of the claimed invention at the time the application was filed. The Examiner contends that only those monoclonal antibodies specifically disclosed in the specification, and not the claimed genus encompassing them, are adequately described, asserting that the several specified monoclonal antibodies are not representative of the genus. The Examiner also has asserted that a genus defined only by functional activity is not adequate to meet the written description requirement, implying that such is the case here. Moreover, the Examiner has stated that reproduction of an identical cell line and antibody is

an extremely unpredictable event, implying that such reproduction is necessary for the practice of the instant invention. Finally, the Examiner refers to the Enzo case as supporting the need for a deposit to satisfy the written description requirement in this case.

In response, Applicants respectfully traverse the Examiner's rejection. First, Applicants believe that the amendments to claims 25 and 26 will help the Examiner to better understand the claimed invention, particularly with respect to what the monoclonal antibodies and fragments will bind. Given this clarification, one of ordinary skill in the art will readily appreciate that the several specific disclosed monoclonal antibodies are fully representative of the claimed genus. The claim amendments help to point out that the members of the claimed genus of monoclonal antibodies and antibody fragments are not merely defined by functional activity. Antibodies which bind to the specific fragments recited in the claims have specific structures that one of ordinary skill would readily recognize. This combination of structural and functional binding characteristics recited in the claims and set forth in the specification provide all necessary information to indicate to one of skill in the art that the Applicants had possession of the invention at the time the application was filed.

With respect to the Examiner's reference to the unpredictability of the reproduction of an identical cell line and antibody, Applicants point out that it is not necessary to produce an identical cell line or an identical antibody to practice the invention.

Finally, Applicants respectfully point out that the Examiner's characterization of the Enzo case is incorrect with respect to the written description requirement. In Enzo, the deposited materials were nucleotide sequences, which the court distinguished from antibodies, which in fact can be sufficiently described by reference to binding characteristics.

In light of the amendments to claims 25 and 26, and the remarks set forth above, Applicants respectfully maintain that the claims are fully described in the specification, and that one of ordinary skill in the art would readily recognize that the Applicants had possession of the claimed invention at the time the application was filed. Accordingly, Applicants respectfully request that the Examiner

reconsider and withdraw the rejection of claims 5, 8, 25, and 26 under 35 U.S.C. §112, first paragraph, written description.

Enablement

The Examiner rejected claims 5, 8, 25, and 26 under 35 U.S.C. §112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to make and/or use the invention. The Examiner stated that the specification lacks complete deposit information for the deposit of the specified monoclonal antibodies, namely 2H9, 6F9, 3G8, 1C4, and 3H8. The Examiner continues to assert that it is not clear that the properties of the specified monoclonal antibodies are known and publicly available, or can be isolated without undue experimentation, and thus, a suitable deposit is required.

In response, Applicants respectfully traverse the Examiner's rejection. Applicants assert that the claims, as amended, are fully enabled by the specification. One of ordinary skill in the art would readily be able to produce antibodies of the claimed genus without undue experimentation, simply by following the teachings of the specification. Techniques to produce such antibodies have been available since before the present invention, as can be seen, for example, in Garnero, et al., Measurement of Serum Osteocalcin with a Human-specific Two-site Immunoradiometric Assay, J. Bone and Mineral Res., Vol. 7, No. 12 (1992) and Garnero, et al., Characterization of Immunoreactive Forms of Human Osteocalcin Generated *in vivo* and *in vitro*, J. Bone and Mineral Res., Vol. 9, No. 2 (1994). Copies of these references are enclosed for the Examiner's convenience. Moreover, as evidenced by the facility of the production of the disclosed antibodies of the claimed genus, producing any antibody of the claimed genus would not require undue experimentation, particularly in light of the teaching of the specific fragments to which the claimed antibodies bind.

In view of the amendments to the claims and the above remarks, Applicants maintain that the claims, as amended, are fully enabled by the specification. Thus, Applicants respectfully request that the Examiner reconsider and withdraw the enablement rejection.

In view of the above amendments and remarks, it is believed that the claims satisfy the requirements of the patent statutes, are patentable over the prior art, and fully address the Examiner's concerns as set forth in the December 17, 2002 Office Action. Reconsideration of the instant application and early notice of allowance are requested. The Examiner is invited to telephone the undersigned if it is deemed to expedite allowance of the application.

RESPECTFULLY SUBMITTED,			
NAME AND REG. NUMBER	Patrick T. Skacel, Reg. No. 47,948		
SIGNATURE		*DATE*	March 17, 2003

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Attachments: Marked-up Copy of Amended Claims
Two references

115A.am6

Marked-Up Copy of the Amended Claims

25 (amended). A monoclonal antibody or recombinant antibody fragment which binds a human gamma-carboxylated osteocalcin fragment, [wherein said monoclonal antibody or recombinant antibody fragment has the specificity to epitopes that have been identified on said gamma-carboxylated fragment of osteocalcin,] wherein said human gamma-carboxylated osteocalcin fragment is selected from the group consisting of

i) a fragment which spans from amino acid in position 7 to amino acid in position 30 of the amino acid sequence set forth in SEQ ID NO:2 in which all three glutamic acids in positions 17, 21 and 24 of said sequence are gamma-carboxylated, and

ii) a fragment which spans from amino acid in position 6 to amino acid in position 30 of the amino acid sequence of SEQ ID NO:2 in which all three glutamic acids in the positions 17, 21 and 24 of said sequence are gamma-carboxylated[.],

and wherein said monoclonal antibody or recombinant antibody fragment has the specificity to epitopes that have been identified on said gamma-carboxylated fragment of osteocalcin.

26 (amended). A non-competitive immunoassay for quantitative determination of a gamma-carboxylated osteocalcin fragment in a sample comprising contacting the sample with two monoclonal antibodies or recombinant antibody fragments which bind said gamma-carboxylated osteocalcin fragment, measuring bound monoclonal antibody or recombinant antibody fragment, and comparing said amount with an amount measured in a sample having a known quantity of said gamma-carboxylated osteocalcin fragment, [wherein said monoclonal antibody or recombinant antibody fragment has the specificity to epitopes that have been identified on said gamma-carboxylated fragment of osteocalcin,] wherein said gamma-carboxylated osteocalcin fragment is selected from the group consisting of

i) a fragment which spans from amino acid in position 7 to amino acid in position 30 of the amino acid sequence set forth in SEQ ID NO:2 in which all three glutamic acids in positions 17, 21 and 24 of said sequence are gamma-carboxylated, and

ii) a fragment which spans from amino acid in position 6 to amino acid in position 30 of the amino acid sequence of SEQ ID NO:2 in which all three glutamic acids in the positions 17, 21 and 24 of said sequence are gamma-carboxylated[.], and

wherein said monoclonal antibody or recombinant antibody fragment has the specificity to epitopes that have been identified on said gamma-carboxylated fragment of osteocalcin.

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Measurement of Serum Osteocalcin with a Human-Specific Two-Site Immunoradiometric Assay

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ABSTRACT

We developed a sensitive and specific two-site radioimmunoassay (IRMA) for human osteocalcin using human osteocalcin as a standard and two monoclonal antibodies raised against human osteocalcin purified from human cortical bone, a solid-phase anti-25–37 region and a tracer anti-5–13 sequence of the molecule. A wide range of osteocalcin levels (up to 300 ng/ml) can be measured with a sensitivity of 0.4 ng/ml. The intra- and interassay coefficients of variation are less than 4 and 6%, respectively. The recovery of human osteocalcin from serum samples ranges from 96 to 103%. IRMA was linear for serial sample dilutions in a wide range of serum osteocalcin levels, even in patients with chronic renal failure on hemodialysis. Depletion of serum in intact osteocalcin demonstrated that IRMA detects, in addition to the intact peptide, a large N-terminal midregion fragment that represents about 50% of total osteocalcin levels in normals and patients with Paget's disease and up to 75% in patients with chronic renal failure. This large fragment, previously unrecognized because it cannot be distinguished from intact osteocalcin with gel filtration chromatography, is not generated in vitro by incubation of the serum up to 26 h. We measured osteocalcin in the serum of 309 healthy adults (180 men and 129 women, age range 20–95 years), 36 patients with Paget's disease, 12 patients with primary hyperparathyroidism, 70 patients with chronic renal failure on hemodialysis, and 10 patients on corticosteroid therapy, simultaneously with human IRMA and with a conventional radioimmunoassay (RIA) based on bovine reagents. A tight correlation ($r = 0.889$) was observed between the two assays in the normal population, but the values obtained with IRMA were about threefold higher (mean 23.3 ± 10.5 versus 7.5 ± 3.4 ng/ml) than those obtained with RIA. Reported as Z scores, that is, number of standard deviations from the predicted normal mean adjusted for sex and age, these two assays (IRMA and RIA) gave concordant results in patients with Paget's disease (4.05 ± 6.21 versus 2.41 ± 2.53), primary hyperparathyroidism (4.14 ± 7.17 versus 2.13 ± 2.28), chronic renal failure (25.32 ± 24.49 versus 6.93 ± 5.48), and glucocorticoid treatment (−1.48 ± 0.78 versus −1.11 ± 0.57). However, IRMA was more discriminant from controls for all these metabolic bone diseases because the absolute values of mean Z scores with IRMA were significantly higher than those obtained with the RIA ($p < 0.05$–0.0001). We conclude that this new human-specific IRMA of osteocalcin may be more sensitive than bovine RIA for the clinical investigation of metabolic bone diseases.

INTRODUCTION

Osteocalcin, also called bone gla protein (BGP), is one of the major noncollagenous proteins of bone matrix produced by osteoblasts.[1–3] This molecule of 49 amino acids contains three residues of γ-carboxyglutamic acid. Osteocalcin binds to hydroxyapatite with high affinity, and it is closely associated with bone matrix. Osteocalcin is chemotactic for many cells[4] and may play a role in the recruitment of osteoclasts,[5] but its precise physiologic function remains unclear. A fraction of newly synthesized osteocalcin is released in blood, where it can be measured

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by radioimmunoassay.[6] Numerous studies have shown that serum osteocalcin is a sensitive marker of bone metabolism in various metabolic bone diseases, such as osteoporosis, primary hyperparathyroidism and hyperthyroidism, and renal osteodystrophy.[6-11] Studies correlating serum osteocalcin with the kinetic and histomorphometric evaluation of bone resorption and formation have shown that serum osteocalcin is a specific index of bone formation whenever resorption and formation are dissociated.[12-18]

Radioimmunoassays that have been developed by several groups, as well as commercial kits, most often utilize bovine osteocalcin as tracer, standards, as well as immunogen for the production of antibodies. Human and bovine osteocalcin differ by 5 amino acids out of a total of 49. The precise antigenic determinants of the osteocalcin molecule have not been clearly identified, but many of these antisera recognize the carboxy-terminal region of the molecule, which has been shown to be identical in both human and bovine osteocalcin.[19] Whatever the epitope recognized by these antisera, most if not all of these assays do not exhibit a 100% cross-reactivity with human osteocalcin, resulting in poor dilution curves with some serum samples.[20,21] In 1988, Taylor et al. developed the first radioimmunoassay against a midmolecular epitope of human osteocalcin involving a region that differs between human and bovine osteocalcin.[22] Preliminary results suggested that this assay may be more sensitive than conventional bovine assays to assess bone turnover in such diseases as Paget's disease and hyperparathyroidism.[22]

Serum osteocalcin appears to circulate mainly as the intact protein, but osteocalcin fragments have also been identified, especially in patients with chronic renal failure and secondary hyperparathyroidism.[23] It is tempting to speculate that such fragments may be released into the circulation during osteoclastic bone resorption, but a recent study using a single antibovine osteocalcin monoclonal antibody has revealed that the most abundant of these fragments is not related to resorption.[24] This fragmentation pattern undoubtedly contributes to the variability between assays and complicates the clinical interpretation of results in various disease states.[23]

To overcome these limitations, we developed a specific and sensitive two-site radioimmunoassay for human osteocalcin, and the results obtained in normal adults and in patients with metabolic bone disease were compared to those obtained with a conventional radioimmunoassay based upon bovine reagents and a rabbit antiserum.

MATERIALS AND METHODS

Purification of human osteocalcin

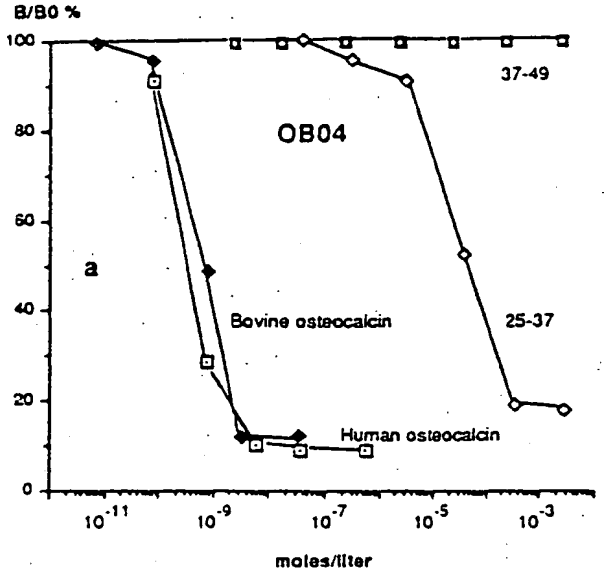
Human osteocalcin was isolated from femoral diaphysis bone taken from a 26-year-old male. Human bone was finely ground in liquid nitrogen, defatted with ice-cold acetone, and washed in ice-cold pure water. Dried bone powder was resuspended in 0.5 M EDTA, pH 7.4, with protease inhibitors (0.2 M ϵ-aminocaproic acid, 0.01 M benzamidine, 0.001 M phenylmethanesulfonyl fluoride (PMSF), and 0.02 M *N*-ethylmaleinimide) and extracted for 24 h at 4°C with constant stirring as previously described.[25] After centrifugation, the supernatant extract was desalted on Sephadex G25 (Pharmacia, Sweden), equilibrated with 50 mM NH_4HCO_3, and freeze dried. The extract was resuspended in 0.5 M phosphate-buffered saline (PBS) with protease inhibitors and applied on an immunoaffinity column using an anti-25-37 bovine osteocalcin antibody coupled to CNBr-activated Sepharose (Pharmacia, Sweden) and eluted with 0.1 M glycine HCl, pH 2.5. Under these conditions 1.8 mg human osteocalcin was obtained from 30 g human cortical bone. The purity of human osteocalcin was controlled by sodium dodecyl sulfate gel analysis and amino acid analysis.

Preparation of monoclonal antibodies to human osteocalcin

Female Balb/C mice and Lou/c rats were immunized by monthly intraperitoneal injections of 10–50 μg purified human peptide coupled or not with pig thyroglobulin emulsified in complete Freund's adjuvant (initial injection) or alugel (all subsequent boosts). Splenocytes were fused 4 days after an intravenous injection of immunogen with mouse P_3 X63 Ag 8.653 myeloma cells, as previously described.[26] Immunized animal sera and hybridoma supernatants were tested by radioimmunoassay using either human or bovine osteocalcin as a tracer. Two families of antibodies were obtained: those that recognized both tracers were classified as anti-C-terminal osteocalcin, and those that recognized human osteocalcin only as anti-N-terminal osteocalcin since differences between human and bovine osteocalcin occur only in the first 19-amino acids.[19] Mouse (OB04) and rat (OR06) monoclonal antibodies were selected for use in a sandwich radioimmunoassay. Mouse monoclonal antibody OS35 was used to study the specificity of the two-site radioimmunoassay of human osteocalcin (IRMA).

Characterization of monoclonal antibodies

We tested the ability of human and bovine osteocalcin and of synthetic peptides 5-13, 25-37, and 43-49 (Neosystem, France) and 7-19 and 37-49 (Bachem, Switzerland) to compete with osteocalcin for the binding on monoclonal antibodies OB04, OR06, and OS35. Purified monoclonal antibodies (200 μl) diluted at the titer (antibody dilution that binds 50% of ^{125}I-osteocalcin) in 50 mM Tris, 2 mM $CaCl_2$, and 0.3% bovine serum albumin (BSA) were incubated overnight at room temperature with 100 μl osteocalcin and 100 μl of different amounts of human or bovine osteocalcin or synthetic peptides. Immune complexes were precipitated by adding a mixture of sheep antimouse (for OB04 and OS35) or antirat (for OR06) immunoglobulin antiserum and normal human serum. After incubation for 15 minutes at room temperature, 1 ml polyethylene glycol 6000 (6% in 0.02 M Tris, pH 8.5) was added and samples were centrifuged for 15 minutes at 2300 × *g*. Bound osteocalcin was determined by counting in a gamma counter (Crystal II, Packard).



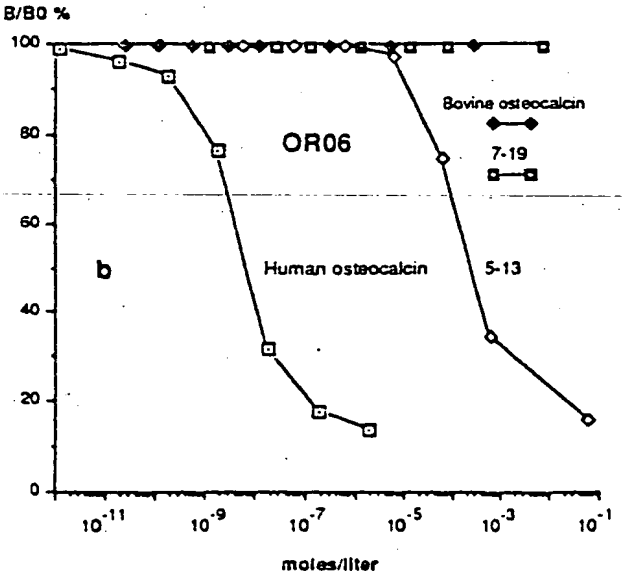


FIG. 1. Epitope specificity of the monoclonal antibodies investigated (OB04 and OR06). (a) For OB04, binding of [^{125}I]osteocalcin is inhibited by human and bovine osteocalcin (IC_{50} = 6.10-9 M) and by the synthetic 25-37 peptide (IC_{50} = 4.10-5 M), but not by the synthetic 37-49 peptide. (b) For OR06, binding of [^{125}I]osteocalcin is inhibited by human osteocalcin (IC_{50} = 8.10-9 M) and by the synthetic 5-13 peptide (IC_{50} = 2.10-4), but not by bovine osteocalcin or synthetic 7-19 peptide. IC_{50}: concentration of displacers (human, bovine osteocalcin, and synthetic peptides) inhibiting 50% of [^{125}I]osteocalcin binding.

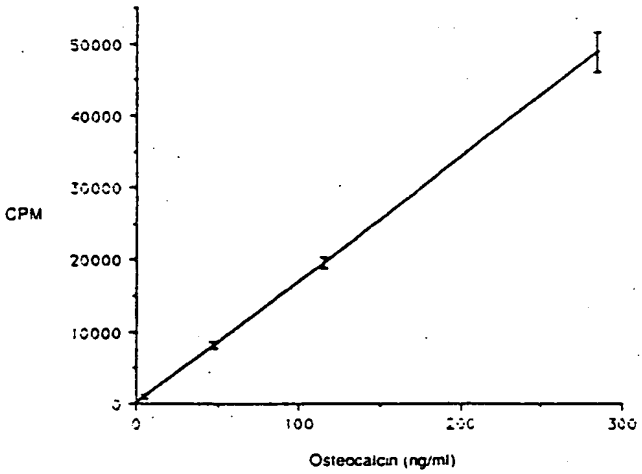


FIG. 2. Standard curve of the two-site radioimmunoassay for human osteocalcin. CPM, counts per minute.

Osteocalcin radioimmunoassays

Two-Site Immunoassay of Human Osteocalcin: Mouse monoclonal antibody OB04 was adsorbed on a solid phase (ELSA; CIS Biointernational, France). Rat monoclonal antibody OR06 was radioiodinated as previously described.[27] The solid phase, 50 µl standards (human osteocalcin in 50 mM borate, 2 mM $CaCl_2$, and 1% BSA), or serum and 300 µl tracer (150 nCi in 50 mM Tris, 2 mM $CaCl_2$, and 0.3% BSA) were incubated for 2 h at room temperature with agitation (CISmix; CIS Biointernational). The unbound tracer was then aspirated, and the solid phase was washed three times with water containing 0.1% Tween 20. Total and bound radioactivity were determined by counting in a gamma counter. The human osteocalcin standard was calibrated by three independent amino acid analyses.

Competitive Assay: Serum osteocalcin levels were determined by a competitive radioimmunoassay (RIA, OSTK-PR; CIS Biointernational) that uses rabbit polyclonal antibodies raised against bovine osteocalcin and bovine osteocalcin as tracer and standards. The sensitivity of the assay is 0.5 ng/ml. The intra- and interassay coefficients of variation were 5 and 7%, respectively. This assay has been used in several studies.[28-32]

Serum gel filtration chromatography

Sera (1-5 ml) from controls and patients were applied to a Sephacryl HR-100 gel. The column was eluted with 0.1 M ammonium bicarbonate, pH 8.0. The osteocalcin immunoreactivity of individual fractions was measured with both radioimmunoassays (IRMA and RIA).

TABLE 1. RECOVERY TEST[a]

Samples	Endogenous osteocalcin (ng/ml)	Added osteocalcin (ng/ml)	Measured value (ng/ml)	Recovery (%)
I	13.3	13.0	25.6	97
	13.3	39.5	50.5	96
	13.3	76.0	87.2	98
	13.3	145.0	155.7	98
II	124.7	13.0	141.8	103
	124.7	39.5	167.5	102
	124.7	76.0	191.8	96
	124.7	145.0	272.3	101

[a]Different known amounts of human osteocalcin standard were spiked into two serum samples from a control (sample I) and from a patient with chronic renal failure (sample II). Recovery is expressed as a percentage of the theoretical osteocalcin value (endogenous + added osteocalcin).

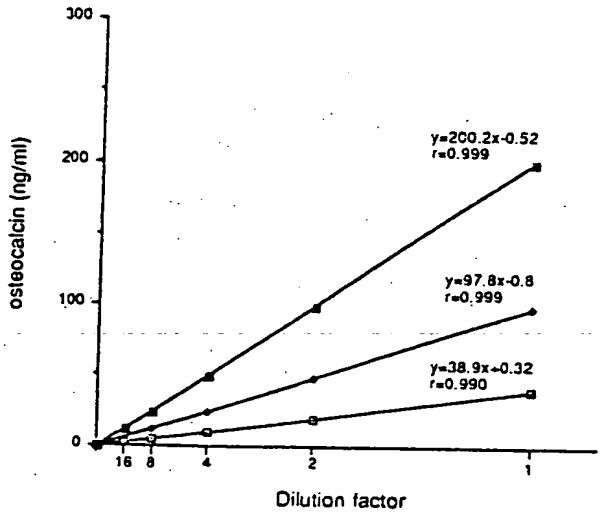


FIG. 3. Plots of osteocalcin levels obtained on serial dilution of serum samples from three patients with chronic renal failure. Serum osteocalcin values are 39.2, 97.1, and 200 ng/ml, respectively.

Depletion of serum in intact human osteocalcin

Pooled sera (400 μl; $N = 10$) from normal subjects, patients with Paget's disease, and patients with chronic renal failure on hemodialysis were incubated for 2 h at room temperature under agitation with a monoclonal antibody recognizing the human osteocalcin sequence 43–49 adsorbed on a solid phase (OS35 monoclonal antibody) or with an antiosteonectin monoclonal antibody as a negative control. The efficacy of the depletion was controlled by assaying depleted sera with an immunoradiometric assay specific for the intact human osteocalcin using monoclonal antibodies anti-43–49 and anti-5–13. A fresh blood sample was measured with the IRMA and with the assay for intact human osteocalcin after 1, 2, 4, 6, 24, 48, and 72 h of incubation at room temperature.

Subjects

Serum osteocalcin was measured in 309 normal adults and 128 patients with metabolic bone disease. None of the normal subjects had a history of any medical disease or was taking drugs known to affect bone metabolism. The 10 patients receiving glucocorticoid therapy had been taking at least 60 mg corticosteroids daily: 6 had rheumatoid arthritis, 2 were treated for bone metastases, and 2 for polyarthritis. Parathyroid adenoma was the cause of the primary hyperparathyroidism in the 12 patients; all had increased serum intact parathyroid hormone (PTH) concentration. A total of 36 patients with symptomatic Paget's disease were referred because of bone pain, and all demonstrated roentgenographic evidence of Paget's disease. A total of 70 patients with chronic renal failure on hemodialysis were recruited.

Statistical analysis

The results are expressed as the mean ± standard deviation (SD). Comparison between serum osteocalcin values obtained with the two radioimmunoassays was evaluated by linear regression analysis. The correlation between serum osteocalcin level and age was assessed by linear and cubic polynomial regression analysis. To measure the sensitivity of the serum osteocalcin assays to discriminate between patients and controls, we used the Z score. This allows comparison of men and women of various age by expressing individual values as the number of standard deviations from the predicted mean for normal subjects obtained from the sex-specific regression between osteocalcin and age. By definition the mean Z score from the sex-specific age regression is 0 in normal subjects. Z scores obtained by both assays were compared by two-tailed t-tests, parametric (Student's) and nonparametric (Mann-Whitney) tests.

RESULTS

Characterization of monoclonal antibodies

To determine the antigenic epitopes on the osteocalcin molecule recognized by OB04, OR06, and OS35, competitive inhibition of [^{125}I]osteocalcin binding by human osteocalcin, bovine osteocalcin, and synthetic peptides was tested. As shown in Fig. 1a, the binding of [^{125}I]osteocalcin on OB04 was inhibited by human and bovine osteocalcin (concentration of displacer inhibiting 50% of [^{125}I]osteocalcin binding, $IC_{50} = 6 \times 10^{-9}$ M) and at high concentration by the 25–37 synthetic peptide ($IC_{50} = 4 \times 10^{-5}$ M) but not by the 37–49 synthetic peptide. For OR06 (Fig. 1b), the binding of [^{125}I]osteocalcin was inhibited by human osteocalcin ($IC_{50} = 8 \times 10^{-9}$ M) and at high concentration by the 5–13 synthetic peptide ($IC_{50} = 2 \times 10^{-4}$ M) but not by bovine osteocalcin or the 7–19 synthetic peptide. The binding of [^{125}I]osteocalcin on OS35 was inhibited by human or bovine osteocalcin and by the 37–49 and 43–49 synthetic peptides but not by the 5–13 and 25–37 synthetic peptides (data not shown). These results suggest that OB04 recognized the 25–37 region, OR06 the 5–13 sequence of the molecule, and OS35 the 43–49 sequence of human osteocalcin.

Two-site radioimmunoassay performance

The standard curve of the two-site assay is shown in Fig. 2. This gives a typical linear range from 0 to 300 ng/ml. The intraassay variation assessed by 15 measurements of three serum samples (mean osteocalcin levels 21.9, 98.4, and 183.9 ng/ml) was less than 4%. The interassay variation evaluated by repeated measurements ($N = 12$) of three serum samples (mean osteocalcin levels 20, 71.9, and 171.4 ng/ml) was less than 6%. The detection limit was 0.4 ng/ml.

Recovery Test: Known amounts of human osteocalcin were added to human serum. The recovery of human osteocalcin from serum samples ranged from 96 to 103% (Table 1).

Dilution Test: The linearity of the method was tested over a wide range of osteocalcin concentrations on serial dilutions of sera from patients with chronic renal failure. In the range of osteocalcin levels examined (40–200 ng/ml) and of dilutions (1:2 to 1:16), the method yielded linear and reproducible results (Fig. 3).

Specificity: Bovine and ovine osteocalcin were not detected by IRMA for concentrations up to 300 ng/ml (data not shown). Gel filtration chromatography of sera from a normal control and a patient with renal osteodystrophy, performed on a Sephacryl HR-100 column (Fig. 4), demonstrated that IRMA detected immunoreactive osteocalcin as a single peak that coelutes with the intact peptide, whereas conventional bovine RIA measured, in addition to the intact molecule, fragments of different sizes in the patient with renal osteodystrophy. After depletion of serum in intact human osteocalcin (Table 2), IRMA still detected

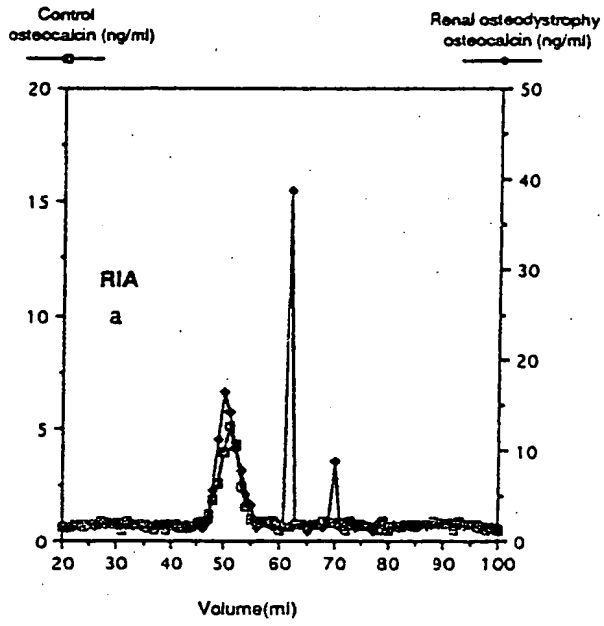


FIG. 4. Chromatography of serum from a normal control subject and from a patient with renal osteodystrophy. Serum samples (1–5 ml) were gel filtered on a Sephacryl HR-100 column eluted with 0.1 M ammonium bicarbonate, pH 8. Immunoreactive osteocalcin was determined by RIA (a) and IRMA (b) in 1 ml effluent.

immunoreactive osteocalcin fragments representing about 50% of the total levels in normal subjects and Paget's and up to 75% of total osteocalcin in chronic renal failure. Competitive RIA recognized fragments (Table 2) that contribute about 30% of the total levels in normal subjects and patients with Paget's disease and chronic renal failure. After incubation for 4 and 6 h at room temperature, the serum levels of intact osteocalcin were slightly decreased by 19 and 8%, respectively, and the serum levels measured by IRMA were not significantly changed (−4 and −10%, respectively).

Correlation with competitive radioimmunoassay

Serum osteocalcin levels were measured in the 309 normal subjects using both RIA and IRMA. A tight correlation ($r = 0.889$) was observed between the two assays, but the values obtained with IRMA were about threefold higher (mean 23.3 ± 10.5 versus 7.5 ± 3.4 ng/ml) than those obtained with the competitive assay (Fig. 5).

Serum osteocalcin levels in normal population

Sex-specific regressions between osteocalcin levels and age are shown in Fig. 6. No significant correlation was found for men (Fig. 6a, $r = 0.1$), and the mean serum osteocalcin level was 19.5 ± 6.1 ng/ml. For women, a positive correlation (Fig. 6b) was found and the regression was best fitted with a polynomial equation (serum osteocalcin, ng/ml = $23.15 - 0.32\text{age} + 0.0061\text{age}^3$, $r = 0.48$, $p < 0.0001$). The values increased significantly after the menopause (30.4 ± 10.8 versus 14.9 ± 3.8 ng/ml in premenopausal women, $p < 0.0001$), as shown in Fig. 7. About 80% of early postmenopausal women (less than 5 years after the menopause) had serum osteocalcin levels that were higher than the upper limit of the normal range for young women (20.2 ng/ml).

TABLE 2. SERUM OSTEOCALCIN LEVELS IN POOLED SERA[a]

	Serum osteocalcin (ng/ml)							
	Intact osteocalcin		*IRMA*		*RIA*		*Fragments (% of total)*	
Serum depletion	*Before*	*After*	*Before*	*After*	*Before*	*After*	*IRMA*	*RIA*
Normal	11.4	0	26.7	15.3	7.1	2.4	57	34
Paget's disease	21.6	0	44.6	26.2	12.9	2.9	59	22
Chronic renal failure	31.0	0	87.5	63.2	19.1	5.3	72	28

[a]Serum samples were from 10 controls, 10 patients with Paget's disease, and 10 patients with chronic renal failure measured before and after depletion in intact osteocalcin with the IRMA and the RIA. Sera were depleted by incubation for 2 h at room temperature with a monoclonal antibody recognizing amino acids 43–49 of the human sequence. The efficacy of depletion is controlled by assaying osteocalcin with a two-site assay specific for intact osteocalcin.

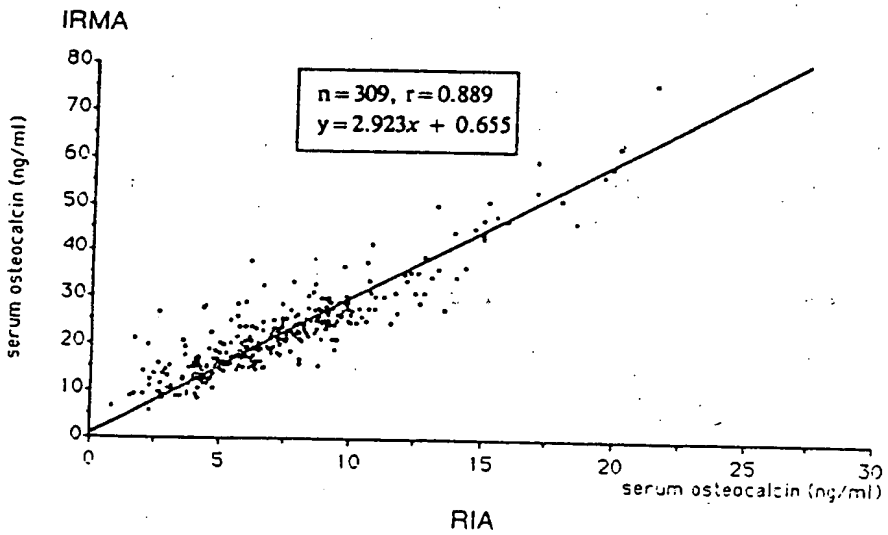


FIG. 5. Correlation study of the two-site (IRMA) and competitive (RIA) osteocalcin radioimmunoassays. Normal serum samples ($n = 309$) were analyzed simultaneously by both assays. Linear regression analysis: $r = 0.889$; $y = 2.923x + 0.655$.

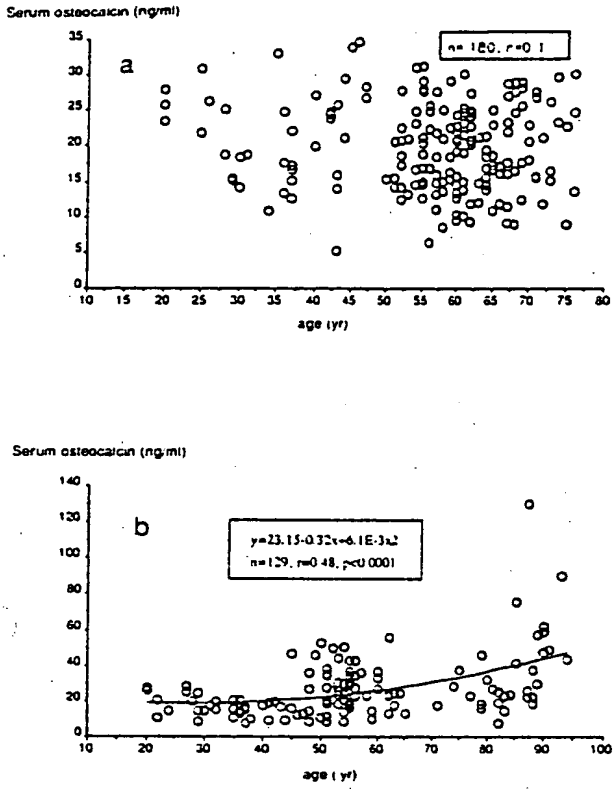


FIG. 6. Relationships between serum osteocalcin level and age in healthy men and women. (a) In normal men, no significant correlation ($r = 0.1$) was observed and the mean serum osteocalcin level was 19.5 ± 6.1 ng/ml. (b) In normal women, the serum osteocalcin level was correlated with age: $y = 23.15 - 0.317age + 0.0061age^2$ ($r = 0.48$, $p < 0.0001$).

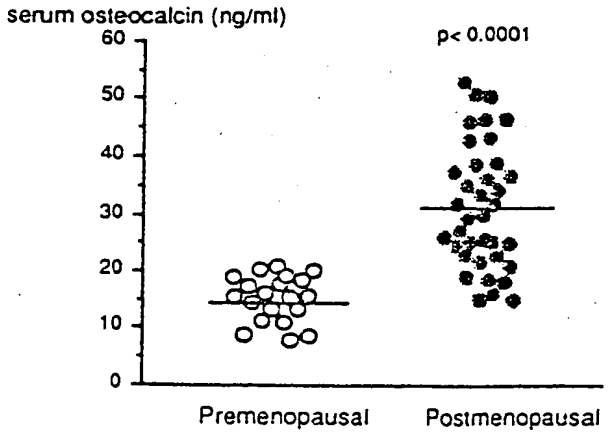


FIG. 7. Individual serum osteocalcin values in 20 premenopausal (range 35–45 years) and in 37 early postmenopausal women (less than 5 years after the menopause). Mean values were 14.9 ± 3.8 and 30.4 ± 10.8 ng/ml, respectively ($p < 0.0001$ by unpaired t-test).

Serum osteocalcin levels in patients with metabolic bone disease

Serum osteocalcin levels were measured in patients with metabolic bone disease using both IRMA and RIA. They were increased in conditions associated with high bone turnover (primary hyperparathyroidism, Paget's disease, and chronic renal failure) and decreased in patients treated by corticosteroids (Table 3). The mean Z scores and the significance of their deviation from normal are shown in Table 4; individual Z score values for IRMA are given in Fig. 8. Both assays gave concordant results in all metabolic bone disorders. The mean Z score observed with IRMA were significantly higher in primary hyperparathyroidism, Paget's disease, and chronic renal failure on hemodialysis and lower in patients on glucocorticoid treatment than the mean Z score obtained with RIA.

DISCUSSION

We have developed a specific two-site radioimmunoassay for human osteocalcin using human bone osteocalcin as a standard and two monoclonal antibodies raised against human osteocalcin. OB04, the antibody adsorbed on the solid phase, is directed against an epitope closed to the C-terminal region of the molecule (amino acids 25–37). However, OB04 recognized the 25–37 synthetic peptide with a low affinity. This is probably due to the three-dimensional structure of the native molecule, notably the disulfite loop (Cys^{23}-Cys^{29}) not present in the peptide. OR06, used as a tracer, is directed against the N-terminal part of the molecule since it does not recognize bovine osteocalcin. The synthetic 7–19 peptide is not detected, whereas the 5–13 peptide can be recognized by OR06 but with a very low affinity, suggesting that it contains only a portion of the antibody binding site. Depletion of serum in intact human osteocalcin demonstrated that this two-site assay detected a large fragment present in normal and pathologic sera in addition to the intact molecule. This N-terminal midregion fragment is large, because it is recognized by IRMA using monoclonal antibodies directed against two distant epitopes (5–13 and 25–37) and since chromatography sieve on HR-100 cannot distinguish it from the intact peptide (Fig. 4). The bovine competitive assay measured, in addition to the intact molecule, fragments of smaller size detected by chromatography that were not recognized by IRMA (Fig. 4). Our experiment measuring intact and fragmented osteocalcin after various incubation times suggests that the large fragment recognized by IRMA is not only generated by proteolytic cleavage in vitro but is also generated in vivo, possibly by osteoblastic synthesis. Further investigations are necessary to characterize the structure and origin of this N-terminal midregion fragment and to investigate its potential clinical significance.

This human-specific IRMA is fast (2 h), and the intra- and interassay coefficients of variation are less than 4 and 6%. The standard curve is linear from 0.4 to 300 ng/ml, a range that compares favorably with the range obtained with competitive assays and with the two-site enzyme immunoassay for γ-carboxylated osteocalcin recently developed by Koyama et al.[34] In addition, osteocalcin can be

TABLE 3. SERUM OSTEOCALCIN LEVELS IN CONTROLS AND PATIENTS WITH METABOLIC BONE DISEASE[a]

Subjects	Male/female	Age (range)	Serum osteocalcin (mean ± SD) IRMA	Serum osteocalcin (mean ± SD) RIA
Control	309 (180/129)	55 ± 15 (20–95)	23.8 ± 10.5	7.5 ± 3.4
Glucocorticoid treatment	10 (5/5)	57 ± 10 (41–75)	10.7 ± 7.6	4.1 ± 3.4
Primary hyperparathyroidism	12 (1/11)	66 ± 7 (56–78)	71.9 ± 77.3	19.4 ± 10.6
Paget's disease	36 (17/19)	69 ± 8 (47–82)	50.2 ± 37.4	16.1 ± 6.8
Chronic renal failure	70 (23/47)	46 ± 9 (31–70)	193.7 ± 165.5	24.9 ± 11.2

[a]Results are expressed as the mean ± SD.

TABLE 4. SERUM OSTEOCALCIN LEVELS IN METABOLIC BONE DISEASES

Subjects	Patients	Z score (serum osteocalcin)[a] IRMA Mean	IRMA p[b]	RIA Mean	RIA p[b]	p[c]
Glucocorticoid treatment	10	−1.48 ± 0.78	<0.0001	−1.11 ± 0.57	<0.0001	<0.05
Primary hyperparathyroidism	12	4.14 ± 7.17	<0.005	2.13 ± 2.28	<0.005	<0.05
Paget's disease	36	4.05 ± 6.21	<0.0001	2.41 ± 2.53	<0.0001	<0.05
Chronic renal failure	70	25.21 ± 24.49	<0.0001	6.93 ± 5.48	<0.0001	<0.0001

[a]Z score: deviation from predicted mean for normal subjects obtained from the sex-specific regression equation that predicted the serum concentration as a function of age.
[b]Significance of difference from normal subjects.
[c]Significance of difference between the two-site assay (IRMA) and the competitive assay (RIA).

determined in all human serum samples, even in patients with chronic renal failure after appropriate dilutions, since the linearity of the method, tested over a wide range of osteocalcin levels and dilutions, was very good (Fig. 3) and the mean recovery of human osteocalcin from serum samples was 99 ± 2.7% (Table 1).

We observed a good correlation between the osteocalcin levels measured with the two-site assay and those obtained with the conventional bovine competitive radioimmunoassay (r = 0.889), but the values with IRMA were about threefold higher than with RIA. We believe that this discrepancy is related to two additive phenomena. First is the incomplete cross-reactivity between human and bovine osteocalcin. Heterologous RIA are based upon competition between labeled bovine osteocalcin and serum human osteocalcin for antibovine osteocalcin antiserum. Serum human osteocalcin is likely to have less affinity for antiserum than bovine osteocalcin used as immunogen and standard, resulting in an underestimation of serum intact human osteocalcin. Indeed, measurement of intact serum osteocalcin, which is the difference between the measurement before and after depletion, was 4.7 ng/ml with bovine RIA and 11.4 ng/ml with IRMA in the pool of sera from healthy adults (Table 2). Second, IRMA and RIA detect osteocalcin fragments that contribute differently to circulating osteocalcin levels. IRMA recognizes a large N-terminal midregion fragment representing 57% of the total level in normal serum, whereas the small osteocalcin fragments detected by RIA represent only 34% of the total level (Table 2).

As previously reported by most studies using conventional competitive assays,[35–37] serum osteocalcin levels were not correlated with age in normal men. This result obtained in a cross-sectional study must be considered cautiously. In fact, Orwoll et al.[37] showed no correlation between serum osteocalcin levels and age in a cross-sectional study, whereas in the same normal male population, serum osteocalcin values increased in a longitudinal study during a 3 year interval. In healthy women, we have shown that serum osteocalcin levels increased with aging, as previously published.[35,38,39] Previous studies have shown that this increase is largely due to the effect of the menopause[38–40] and reflects an increase in bone turnover. With our IRMA, serum osteocalcin in early postmenopausal women was 51% higher than in premenopausal women, and 80% had levels higher than the upper limit of those observed in premenopausal. The increase in osteocalcin levels in elderly normal women (more than 75 years old) is more complex; it can be partly explained by an increase in the noncarboxylated fraction of circulating osteocalcin.[41]

Results in patients with metabolic bone diseases are consistent with previous reports showing that serum osteocal-

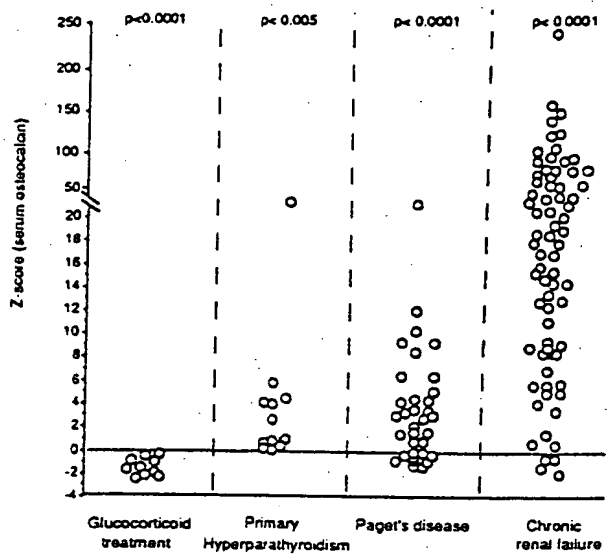


FIG. 8. Individual serum osteocalcin values (*Z* scores) in patients treated with glucocorticoids, in patients with primary hyperparathyroidism, in patients with Paget's disease, and in patients with chronic renal failure measured by the two-site radioimmunoassay (IRMA). *Z* scores were determined from the predicted mean for normal subjects obtained from the sex-specific regression equation that predicted serum osteocalcin concentrations as a function of age. Significant difference *p* from normal subjects is indicated.

cin levels are increased in patients with primary hyperparathyroidism,[6,7] Paget's disease,[6,8,42,43] and chronic renal failure on hemodialysis[14,16,31] and decreased in patients treated by corticosteroids.[44,45] Interestingly, the discrimination (*Z* score) between serum osteocalcin levels measured in these bone diseases and serum levels in the control population was significantly enhanced using IRMA rather than bovine RIA (Fig. 8 and Table 4). This enhanced sensitivity was especially striking in patients with chronic renal f[illegible]e, with mean *Z* scores of 25 for IRMA and 7 with RIA. Such an increased sensitivity with a homologous sandwich assay was previously documented for the measurement of serum parathyroid hormone in patients with primary hyperparathyroidism.[46]

In conclusion, we have described a human-specific two-site IRMA for serum osteocalcin that has several advantages over the conventional bovine osteocalcin-based competitive RIA. This assay is fast and highly reproducible and provides adequate dilution curves for all tested sera. Although this IRMA recognizes both intact osteocalcin and a large N-terminal midregion fragment, results obtained in patients with various metabolic bone diseases suggest that this IRMA is more sensitive than conventional bovine osteocalcin-based RIA to detect an increase (or a decrease) in bone turnover. Finally, this IRMA detected a twofold increase in serum osteocalcin after the menopause, suggesting that it might be useful to investigate the changes in bone turnover associated with osteoporosis.

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REFERENCES

1. Hauschka PV, Lian JB, Gallop PM 1975 Direct identification of the calcium-binding amino acid gamma-carboxyglutamate in mineralized tissue. Proc Natl Acad Sci USA 72:3925–3929.
2. Price PA, Otsuka D, Poser JW, Kristaporis J, Raman N 1976 Characterization of a gamma-carboxyglutamic containing protein from bone. Proc Natl Acad Sci USA 73:1447–1451.
3. Beresford JN, Gallagher JA, Poser JW, Russel RGG 1984 Production of osteocalcin by human bone cells in vitro. Effects of $1,25(OH)_2D_3$, $24,25(OH)_2D_3$, parathyroid hormone, and glucocorticoids. Metab Bone Dis Rel Res 5:229–234.
4. Mundy GR, Poser JW 1983 Chemotactic activity of the gamma carboxyglutamic acid containing protein in bone. Calcif Tissue Int 385:164–168.
5. Glowacki J, Lian JB 1987 Impaired recruitment and differentiation of osteoclast progenitors by osteocalcin deplete bone implants. Cell Differentiation 21:247–254.
6. Price PA, Purthermore JG, Deftos IJ 1980 New biochemical marker for bone metabolism: Measurement by radioimmunoassay for gla protein in the plasma of normal subjects and patients with bone disease. J Clin Invest 66:878–883.
7. Delmas PD, Wahner HW, Mann K, Riggs BL 1983 Assessment of bone turnover in postmenopausal osteoporosis of serum bone gla protein. J Lab Clin Med 102:470–476.
8. Gundberg CM, Lian JB, Gallop PM, Steinberg JJ 1983 Urinary γ carboxyglutamic acid serum osteocalcin as bone markers: Studies in osteoporosis and Paget's disease. J Clin Endocrinol Metab 57:1221–1225.
9. Deftos LJ; Parthermore JG, Price PA 1982 Changes in plasma bone gla protein during treatment of bone disease. Calcif Tissue Int 34:121–124.
10. Slovik DM, Gundberg CM, Neer RM, Lian JB 1984 Clinical evaluation of bone turnover by serum osteocalcin measurements in a hospital setting. J Clin Endocrinol Metab 59:228–231.
11. Delmas PD, Wilson DM, Mann KG, Riggs BL 1983 Effects of renal function on plasma levels of bone gla protein. J Clin Endocrinol Metab 57:1028–1030.
12. Brown JP, Delmas PD, Malaval L, Edouard C, Chapuy MC, Meunier PJ 1984 Serum bone gla-protein: A specific marker for bone turnover in postmenopausal osteoporosis. Lancet 1: 1091–1093.
13. Charles P, Poser JW, Mosekilde L, Jensen FT 1985 Estimation of bone turnover evaluated by ^{47}Ca-kinetics. Efficiency of serum bone gamma-carboxyglutamic acid containing protein, serum alkaline phosphatase, and urinary hydroxyproline excretion. J Clin Invest 76:2254–2258.
14. Malluche HH, Faugere MC, Fanti P, Price PA 1984 Plasma levels of bone gla-protein reflect bone formation in patients on chronic maintenance dialysis. Kidney Int 26:869–874.
15. Delmas PD, Malaval L, Arlot ME, Meunier PJ 1985 Serum bone gla-protein compared to bone histomorphometry in endocrine diseases. Bone 6:339–341.
16. Charhon SA, Delmas PD, Malaval L, Chavassieux PM, Arlot M, Chapuy MC, Meunier PJ 1986 Serum bone gla protein in renal osteodystrophy: Comparison with bone histo-

morphometry. J Clin Endocrinol Metab 63:892–897.
17. Eastell R, Delmas PD, Hodgson SF, Eriksen EF, Mann KG, Riggs BL 1988 Bone formation rate in older normal women: Concurrent assessment with bone histomorphometry, calcium kinetics, and biochemical marker. J Clin Endocrinol Metab 67:741–748.
18. Delmas PD 1988 Biochemical markers of bone turnover in osteoporosis. In: Riggs BL, Melton LJ (eds.) Osteoporosis: Etiology, Diagnosis and Management. Raven Press, New York, p. 297.
19. Poser JW, Esch FS, Lig NC, et al. 1980 Isolation and sequence of vitamin K-dependent protein from human bone. J Biol Chem 225:8685–8691.
20. Delmas PD 1980 Biochemical markers of bone turnover for the clinical assessment of metabolic bone disease. Endocrinol Clin North Am 19:1–18.
21. Delmas PD, Christiansen C, Mann KJ, Price PA 1990 Bone gla protein (osteocalcin) assay standardization report. J Bone Miner Res 5:5–11.
22. Taylor AK, Linkhart SG, Mohon S, Baylink DJ 1988 Development of a new radioimmunoassay for human osteocalcin: Evidence for a midmolecule epitope. Metabolism 37:872–877.
23. Gundberg C, Weinstein RS 1986 Multiple immunoreactive forms of osteocalcin in uremic serum. J Clin Invest 77:1762–1767.
24. Tracy RP, Andrianorivo A, Riggs BL, Mann KG 1990 Comparison of monoclonal and polyclonal antibody-based for osteocalcin: A study of sources of variation in assay results. J Bone Miner Res 5:451–461.
25. Demiaux B, Malaval L, Chenu C, Delmas PD 1990 Rapid extraction of non collagenous proteins from human bone. J Bone Miner Res 5(Suppl. 2):abstract 365.
26. Stahli C, Stalhelin T, Miggiano V 1983 Spleen cell analysis and optimal immunization for high frequency production of specific hybridomas. Methods Enzymol 92:26–36.
27. Greenwood FG, Hunter WN, Glover JS 1963 The preparation of ^{131}I-labelled human growth hormone. Biochem J 89: 114–120.
28. Tarallo P, Henny B, Fournier B, Siest G 1990 Plasma osteocalcin: Biological variations and reference limits. Scand J Clin Lab Invest 50:649–655.
29. Pietschmann P, Knoflach P, Wolosczczuk W 1991 Increased serum osteocalcin levels in patients with lactase deficiency. Am J Gastroenterol 56:72–74.
30. Pietschmann P, Woloszczuk W, Panzer S, Kyrle P, Smolen J 1988 Decreased serum osteocalcin levels in phenprocoumon-treated patients. J Clin Endocrinol Metab 66:1071–1074.
31. Sebert JI, Ruiz JC, Fournier A, Fardellone P, Guéris J, Marié A, Morinière P, Codet MP, Renaud H 1987 Plasma bone gla-protein: Assessment of its clinical value as an index of bone formation in hemodialyzed patients. Bone Miner 2: 21–27.
32. Bergmann P, Valsamis J, Van Perborgh J, De Schepper J, Van Vliet G 1990 Comparative study of the changes in insulin-like growth factor-I, procollagen-III N-terminal extension peptide, bone gla-protein, and mineral content in children with Turner's syndrome treated with recombinant growth hormone. J Clin Endocrinol Metab 71:1461–1467.
33. Gundberg CM, Lian JB, Gallop PM 1983 Measurement of γ carboxyglutamate and circulating osteocalcin in normal children and adults. Clin Chim Acta 128:1–8.
34. Koyama N, Ohara K, Yokota H, Kurome T, Katayama M, Hino F, Kato I, Akai T 1991 A one step sandwich enzyme immunoassay for γ carboxylated osteocalcin using monoclonal antibodies. J Immunol Methods 139:17–23.
35. Epstein S, Poser J, McClintock R, Johnston C, Bryce G, Hui S 1984 Differences in serum bone gla protein with age and sex. Lancet 1:307–310.
36. Catherwood BD, Marcus R, Madvig P, Cheuny AK 1985 Determinants of bone γ-carboxyglutamic acid containing protein in plasma of healthy aging subjects. Bone 6:9–13.
37. Orwoll ES, Deftos LJ 1990 Serum osteocalcin (BGP) levels in normal men: A longitudinal evaluation reveals an age-associated increase. J Bone Miner Res 5:259–262.
38. Delmas PD, Stenner D, Wahner HW, Mann KG, Riggs BL 1983 Increase in serum bone gamma carboxyglutamic acid protein with aging in women. J Clin Invest 71:1316–1421.
39. Kelly PJ, Pocock NA, Sambrook PN, Eisman JA 1989 Age and menopause-related changes in indices of bone turnover. J Clin Endocrinol Metab 69:1160–1165.
40. Johansen J, Riis BJ, Delmas PD, Christiansen C 1988 Plasma BGP: An indicator of spontaneous bone loss and of the effects of oestrogenous treatment in postmenopausal women. Eur J Clin Invest 18:191–195.
41. Plantalech L, Guillaumont M, Vergnaud P, Leclercq M, Delmas PD 1991 Impairment of gamma carboxylation of circulating osteocalcin (bone gla protein) in elderly women. J Bone Miner Res 11:1211–1216.
42. Delmas PD, Demiaux B, Malaval L, Chapuy MC, Meunier PJ 1986 Serum bone gla protein is not a good marker of bone turnover in Paget's disease of bone. Calcif Tissue Int 38:60–61.
43. Wilkinson MR, Wagstaffe C, Delbridge L, Wiseman J, Posen S 1986 Serum osteocalcin concentrations in Paget's disease of bone. Arch Intern Med 146:268–271.
44. Reid IR, Chapman GE, Fraser TR, Davies AD, Surus AS, Meyer J, Huq NL, Ibbertson HK 1986 Low serum osteocalcin levels in glucocorticoid-treated asthmatics. J Clin Endocrinol Metab 62:379–383.
45. Garrel DR, Delmas PD, Welsh C, et al. 1988 Effect of moderate physical training on prednisone-induced protein wasting: A study of whole bone and bone metabolism. Metabolism 37:257–262.
46. Blind E, Schmidt-Gayk H, Scharlas S, Flentje D, Fisher S, Göhring U, Hiteler W 1988 Two-site assay of intact parathyroid hormone in the investigation of primary hyperparathyroidism and other disorders of calcium metabolism with a mid region assay. J Clin Endocrinol Metab 67:353–360.

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Characterization of Immunoreactive Forms of Human Osteocalcin Generated In Vivo and In Vitro

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ABSTRACT

Three monoclonal antibodies recognizing the 5–13, 25–37, and 43–49 sequence of the human osteocalcin were used in competitive and two-site radioimmunoassays (RIA) to characterize specifically various immunoreactive forms of circulating human osteocalcin. The intact molecule accounts for 36% of total in normals (2.6 nM), 46% in patients with osteoporosis (3.1 nM), and 26% in chronic renal failure (6.9 nM). Four fragment were detected in addition to the intact molecule in the serum of healthy adults and patients with metabolic bone disease. N-terminal, mid, and mid C-terminal fragments were present in minute amounts (each accounting for 5–14% of the total circulating osteocalcin immnoreactivity). In contrast, the N-terminal midfragment, probably resulting from the cleavage around amino acids 43–44, represents about 30% (2 nM) of the total osteocalcin immunoreactive level in normals and patients with osteoporosis and up to 50% (13 nM) in patients with chronic renal failure. This large N-terminal midfragment, representing 75–80% of the intact osteocalcin level, is not lower when the plasma assay is performed immediately after sampling (within 20 minutes at 4°C with proteinase inhibitors), indicating that it circulates in vivo. In addition, this fragment was detected in the supernatant of osteoblastic cells, representing about 28% of the intact peptide. Levels of N-terminal midfragment were not changed after treatment of patients with metabolic bone disease (Paget's disease, reflex sympathetic dystrophy, fibrous dysplasia, and osteoporosis) by bisphosphonate, suggesting that it is not released during bone resorption. The osteocalcin level measured with the two-site immunoradiometric assay specific for the intact molecule or with a conventional bovine RIA was rapidly decreased after incubation of serum at room temperature (−20 and −15%, respectively, after 3 h), whereas the total level of intact osteocalcin plus N-terminal midfragment was not changed. Intact osteocalcin loss can be partially avoided by proteinase inhibitors and by incubating serum at 4°C. In conclusion, we characterized multiple immunoreactive forms of osteocalcin that circulate in addition to the intact molecule, none of them being specifically altered in osteoporosis. The N-terminal midfragment circulates in a large amount, probably resulting from cleavage of the intact molecule in the circulation and/or at peripheral sites. These fragments can also be generated in vitro by proteolytic degradation of the intact molecule. To obtain reliable intact osteocalcin values but also reliable levels measured with conventional competitive RIA, careful control of the sampling conditions is warranted.

INTRODUCTION

OSTEOCALCIN, also called bone gla protein, a 49 amino acid peptide, is a vitamin K-dependent calcium binding protein produced by osteoblasts.[1–4] It constitutes up to 20% of the noncollagenous proteins of bone matrix.[2,5] Although osteocalcin binds tightly to hydroxyapatite,[1] a fraction of newly synthesized molecule is released in blood, where it can be detected by radioimmunoassay (RIA).[6] Osteocalcin is increased in the circulation of patients with metabolic bone disease characterized by high bone turnover[7–12] and can be used as an index of bone forma-

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tion.[12] The serum half-life of osteocalcin is very short (approximately 5 minutes), because it is rapidly metabolized mainly in the kidney[13] and to a lesser extent in the liver.[14] In these tissues, osteocalcin is hydrolyzed by mitochondrial, microsomal, and supernatant fractions.[14] In plasma, some immunoreactive but not characterized osteocalcin fragments have been found in normals,[15] in patients with chronic renal failure,[16,17] and in patients with Paget's disease.[18] Recently we detected a large N-terminal midregion fragment, previously unrecognized, that represents about 50% of the total osteocalcin level measured with a new two-site immunoradiometric assay (IRMA) in normals and patients with Paget's disease of bone and up to 75% in chronic renal failure.[16] The disparity between osteocalcin fragments reported by several studies may be due to the different ability of various antibodies to detect osteocalcin-related peptides or to multiple forms of osteocalcin,[15,19,20] In addition, fragments detected in the serum of patients with high bone turnover have been speculated to be released into the circulation during osteoclastic resorption of the bone matrix,[17] and a Paget's disease-specific fragment was recently reported.[15] Nevertheless, the most abundant of these osteocalcin peptides does not seem to be related to bone resorption,[19] and the significance of these fragments remains unclear. In human osteocalcin, peptide bonds involving arginine residues 19–20 and 43–44 are susceptible to proteolytic hydrolysis.[16,17,21] Peptides 1–19, 20–43, 45–49, 1–43, and 20–49 are therefore likely to be the main product of osteocalcin breakdown.[14]

To characterize serum osteocalcin fragments and their generation, we used three monoclonal antibodies (MoAb) recognizing amino acids 5–13, 25–37, and 43–49 of the human sequence. These MoAb have been used in RIA to detect serum intact osteocalcin and its potential N-terminal, C-terminal, mid, N-terminal-mid, and mid-C-terminal fragments (Fig. 1).

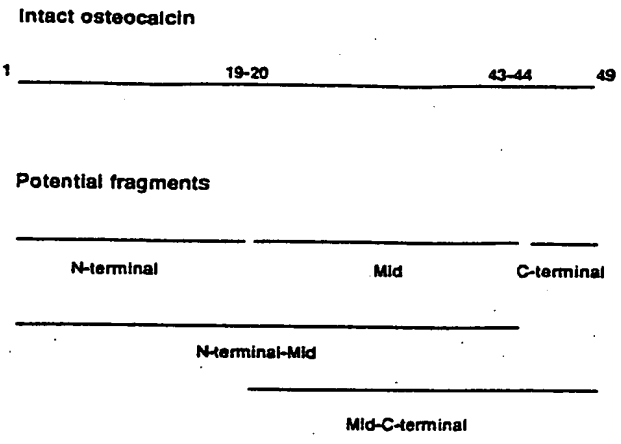

FIG. 1. Intact osteocalcin molecule and potential proteolytic fragments.

MATERIALS AND METHODS

Human osteocalcin standard

Human osteocalcin purified from human cortical bone was used as a standard as previously described.[18] The human osteocalcin standard was calibrated by three independent amino acid analyses.[18]

Monoclonal antibodies

Three MoAb were used to develop six RIA. Mouse (MoAb 43–49 and MoAb 25–37) and rat (MoAb 5–13) MoAb recognized the 43–49, 25–37, and 5–13 sequences of human osteocalcin, respectively, and were obtained as previously described.[18]

Two-site immunoradiometric assays

Three IRMA (A, B, and C) were developed, and the osteocalcin immunoreactive forms detected by these assays are described in Table 1. MoAb directed to the 43–49 sequence (MoAb 43–49, assay A) and MoAb 25–37 (assays B and C) were adsorbed on a porous bead (ELSA; Cisbiointernational, France). MoAb 5–13 (assays A and B) and MoAb 43–49 (assay C) were radioiodinated as previously described.[22] Standard [50 μl; human osteocalcin in 50 mM borate, 2 mM $CaCl_2$, and 1%, bovine serum albumin, BSA)] or serum and 300 μl tracer (830 ng/ml for MoAb 5–13 and 83 ng/ml for MoAb 43–49) were incubated for 2 h at room temperature with agitation (CiSmix; Cisbiointernational, France). The unbound tracer was then aspirated, and the porous bead was washed three times with water containing 0.1% Tween 20. Total and bound radioactivity were determined by counting in a gamma counter (Crystal II; Packard, Downers Grove, IL). The mean counts per minute (cpm) for nonspecific binding were 95.9 ± 7.2, 116.8 ± 9.3, and 281.6 ± 26.4, and the mean bound cpm obtained from normal sera were 3744 ± 152 (% of total radioactivity; 2.03 ± 0.08%), 6558 ± 137 (3.55 ± 0.07%), and 4348 ± 7 (3.67 ± 0.006%), respectively, for assays A, B, and C. The standard curves were linear up to 200 ng/ml (assay A), 300 ng/ml (assay B), and 150 ng/ml (assay C). The intraassay coefficients of variation (two serum samples, $N = 30$) were <3.5, 3.8, and 3.6%, and the detection limits were 0.3, 0.4, and 0.5 ng/ml, respectively, for assays A, B, and C.

Competitive radioimmunoassays

Three RIA (D, E, and F) were developed (Table 2). The MoAb, tracers, and immunoreactive forms of human osteocalcin detected by the assays are described in Table 2. MoAb 5–13 was used to measure intact osteocalcin and N-terminal and N-terminal midfragments with human 1–20 osteocalcin as a tracer (assay D). Intact osteocalcin and mid, N-terminal mid, and mid C-terminal fragments were assayed by RIA using bovine osteocalcin as tracer and MoAb 25–37 (assay E). Assay F (Table 2) detected C-terminal and mid C-terminal fragments in addition to the intact osteocalcin using MoAb 43–49 and human 43–49

osteocalcin coupled to BSA as a tracer. Standard (50 μl; human osteocalcin in 50 mM borate, 2 mM $CaCl_2$, and 1% BSA) or serum, 100 μl MoAb diluted in 50 mM Tris, 150 nM NaCl, 2 mM $CaCl_2$, and 0.3% BSA (initial MoAb concentrations, 80 ng/ml for MoAb 5–13, 5 ng/ml for MoAb 25–37, and 80 ng/ml for MoAb 43–49), pH 8, and 250 μl tracer (20,000 cpm; 1.3, 0.25, and 10 ng/ml, respectively, for assays D, E, and F) were incubated overnight at room temperature. The immune complexes were precipitated by adding a mixture of sheep antimouse (for MoAb 25–37 and MoAb 43–49) or antirat (for MoAb 5–13) immunoglobulin antiserum and normal human serum. After incubation for 15 minutes at room temperature, 1 ml polyethylene glycol 6000 (6% in 0.02 M Tris, pH 8.5) was added and samples were centrifuged for 15 minutes at 2300 × *g*. Bound osteocalcin was determined by counting in a gamma counter, and the concentrations were determined from a spline regression. The maximal bindings (% total radioactivity) were 35.6 ± 0.9, 45.6 ± 0.4, and 11.5 ± 0.2%, and the mean bound radioactivities (% maximal binding) for normal sera were 32.8, 18.2, and 57.5%, respectively, for assays D, E, and F. The intraassay coefficients of variation assessed by repeated measurements (N = 10) of one normal serum sample were 4.0, 4.5, and 5.2%, and the detection limits were 0.081, 0.023, and 0.32 ng/ml, respectively, for assays D, E, and F.

Standard conditions for sampling

To reduce the in vitro proteolytic degradation, blood was taken in tubes containing agar-agar (Vacutainer; Becton-Dickinson), allowed to stand for 60 minutes at 4°C, centrifuged for 10 minutes at 1000 × *g*, and then immediately frozen at −80°C. Under these conditions, the time course between blood drawing and freezing was less than 1 h, 30 minutes. Intact osteocalcin and N-terminal midfragment levels measured in 19 serum samples from normal adults taken under these standard sampling conditions were compared to the levels of six fresh plasma samples (anticoagulated with lithium heparin) from healthy volunteers taken on ice with or without proteinase inhibitors (0.2 M ε-aminocaproic acid, 0.01 M benzamidine, 0.001 M phenylmethanesulfonylfluoride, and 0.02 M *N*-ethylmaleinimide] and assayed within 20 minutes after blood drawing.

Depletion of serum in immunoreactive forms of human osteocalcin

Pooled sera (300 μl; 15 samples for each pool) taken under standard conditions were incubated for 2 h at room temperature under agitation with porous bead (ELSA; Cis biointernational, France) adsorbed with MoAb recognizing different amino acid sequences or with an antiosteonectin

TABLE 1. TWO-SITE IMMUNORADIOMETRIC ASSAYS FOR IMMUNOREACTIVE FORMS OF OSTEOCALCIN[a]

Assay	*Solid-phase MoAb*	*^{125}I tracer MoAb*	*Immunoreactive osteocalcin measured*
A	43–49	5–13	Intact
B	25–37	5–13	Intact N-terminal mid
C	25–37	43–49	Intact Mid C-terminal

[a]Three monoclonal antibodies (MoAb) recognizing the 5–13, 25–37, and 43–40 sequence of human osteocalcin were used to develop assays A, B, and C. Purified human bone osteocalcin was used as standard.

TABLE 2. COMPETITIVE RADIOIMMUNOASSAYS FOR IMMUNOREACTIVE FORMS OF HUMAN OSTEOCALCIN[a]

Assay	*Antibody*	*^{125}I tracer osteocalcin*	*Immunoreactive osteocalcin measured*
D	5–13	Human 1–20	Intact, N-terminal, N-terminal mid
E	25–37	Bovine osteocalcin	Intact, mid, N-terminal mid, mid C-terminal
F	43–49	Human 43–49 BSA	Intact, C-terminal, mid C-terminal

[a]Monoclonal antibodies recognizing the 5–13, 25–37, 25–37, and 43–49 sequence of human osteocalcin sere used in three competitive radioimmunoassays specific for various osteocalcin fragments. Purified human bone osteocalcin was used as standard.

MoAb as a negative control. One bead was used to deplete pooled sera from normals and patients with osteoporosis, whereas six beads were necessary to remove fragments from pooled sera of patients with chronic renal failure. Fragments removed by the depletions are described in Table 3. The efficacy of the depletions performed three times for each pool was controlled by assaying depleted sera with RIA. As shown in Table 3, levels of removed fragments were not detectable except for depletions of pooled sera from patients with chronic renal failure with MoAb 25–37 (assay B still measured 0.09 ± 0.07 nM, whereas the level before depletion was 20.31 nM) and with MoAb 5–13 + MoAb 43–49 (assay B still measured 0.48 ± 0.12 nM with an initial level of 20.31 nM). In all cases, the percentage of removed fragments was >97%.

Characterization of serum immunoreactive osteocalcin forms

Several combinations of depletions and assays described in Table 3 were used to measure osteocalcin immunoreactive forms in pooled serum from healthy volunteers, patients with chronic renal failure on hemodialysis, patients with Paget's disease, and patients with osteoporosis characterized by one or more vertebral fracture. For each of these conditions one pool of 15 samples was studied. Depletions and assays were performed three times for each pool. In addition, intact osteocalcin and N-terminal midfragment were assayed in 11 individual serum from healthy volunteers.

In vitro osteocalcin degradation and N-terminal midfragment generation

A total of 10 normal serum samples were measured with assay A (intact osteocalcin) and assay B (intact osteocalcin plus N-terminal midfragment) after 2 h of incubation at room temperature (with or without proteinase inhibitors). Eight normal human serum samples were assayed with assay A (intact osteocalcin), assay B (intact osteocalcin plus N-terminal midfragment), and RIA (OSTK-PR; Cis biointernational, France), which uses polyclonal antibodies raised against bovine osteocalcin and bovine osteocalcin as tracer and standards after 1, 3, 5, and 24 h of incubation at room temperature and at 4°C. This RIA has been used in several clinical studies.[23–26]

Cell culture

The MG-63 human osteosarcoma cell line was purchased from Flow Laboratories (Puteaux, France). Cells were grown in RPMI medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 1% streptomycin-penicillin. Cells were harvested by trypsin-EDTA treat-

TABLE 3. CHARACTERIZATION OF IMMUNOREACTIVE FORMS OF HUMAN OSTEOCALCIN[a]

Depletion		*Measurement*		*Depletion control*		
					Osteocalcin after depletion	
MoAb	*Removed fragments*	*Assay*	*Fragment*	*Assay*	*Serum*	*Level (nM)*
5–13	Intact N-terminal N-terminal mid	C	Mid C-terminal	A	Normal OP CRF	ND[b] ND ND
25–37	Intact Mid N-terminal mid Mid C-terminal	D	N-terminal	B	Normal OP CRF	ND ND 0.09 ± 0.07
		F	C-terminal	C	Normal OP CRF	ND ND ND
43–49	Intact C-terminal Mid C-terminal	B	N-terminal mid	A	Normal OP CRF	ND ND ND
5–13 + 43–49	Intact N-terminal C-terminal N-terminal mid Mid C-terminal	E	Mid	B + C	Normal OP CRF	ND (assays B and C) ND (assays B and C) 0.48 ± 0.12 (assay B) ND (assay C)

[a]For the depletions, serum was incubated 2 h at room temperature with monoclonal antibodies (MoAb) recognizing different regions of human osteocalcin or with an antiosteonectin MoAb as a negative control. The efficacy of the depletions performed three times for each pool of serum [normal, from patients with osteoporosis (OP), and from patients with chronic renal failure CRF)] was controlled by measuring removed fragments by specific radioimmunoassays. Several combinations of depletions followed by radioimmunoassays were used to measure osteocalcin fragments.

[b]Not detectable.

ment and passaged at a ratio of 1:8 once a week. Human osteoblast-like cells were derived from human cancellous bone explants. The bone specimen was collected from long bone of a male patient undergoing surgery for treatment of traumatic fracture. The bone explant was processed as previously described.[27] Confluent cells (MG-63 or human osteoblast-like cells) were washed twice with a FBS-free growth medium and preincubated with the same medium supplemented with 0.5% BSA and 10 nM 1,25-$(OH)_2D_3$ for 24 h. The incubation started by replacing this medium and was stopped at appropriate times by removing the supernatant stored at −80°C. Intact osteocalcin and N-terminal midfragment were measured in cell culture supernatants with RIA A and B (Table 1).

Effect of treatment with pamidronate on the intact osteocalcin and N-terminal midfragment levels

Intact osteocalcin and N-terminal midfragment were measured in the serum of seven patients with active Paget's disease, eight patients with reflex sympathetic dystrophy, four patients with fibrous dysplasia, and four patients with osteoporosis before and after a 3 day intravenous treatment with pamidronate (60 mg/day, i.e., 180 mg total).

Statistical analysis

Results are expressed as mean ± standard deviation (SD). A nonparametric Mann-Whitney U test was used for comparison of two means, and the Wilcoxon t-test was used for the comparison of paired values.

RESULTS

Osteocalcin immunoreactive forms in the sera of adults and of patients with metabolic bone disease

Intact osteocalcin and fragments were measured in pooled serum from healthy adults and patients with metabolic bone disease using depletions of serum followed by specific RIA. The molar concentration and the proportion of the different osteocalcin immunoreactive species assayed in pooled sera are shown in Table 4. In normal adults, intact osteocalcin and N-terminal midfragment represent the main circulating osteocalcin species at about the same level (2–2.6 nM). Results obtained in 11 individual serum samples from healthy volunteers were similar to those observed for the pool of normal sera (intact osteocalcin, 1.89 ± 0.64 nM; N-terminal midfragment, 1.57 ± 0.55 nM; data not shown). In patients with osteoporosis, the levels of the different immunoreactive forms were similar to those observed for normal subjects (Table 4). Intact osteocalcin and fragments were increased in patients with chronic renal failure (Table 4). The N-terminal midfragment was increased in patients with Paget's disease (4.7 versus 2.14 nM in controls; data not shown), whereas its percentage of intact osteocalcin was not significantly changed (91 versus 83% for controls, data not shown). This large fragment was especially accumulated in patients with chronic renal failure, with a sixfold increase above the normal concentration (13.4 0.36 nM versus 2.14 ± 0.08 nM for normals). In this disease, this peptide represents about 50% of the total osteocalcin level (Table 4) and 193% of the intact osteocalcin values. Exogenous 43–49 osteocalcin synthetic peptide, diluted in assay buffer, was detected by RIA F (Table 2) using MoAb 43–49 and 43–49 osteocalcin BSA as tracer with a sensitivity of 0.32 ng/ml. However, when this peptide was added at different concentrations to serum, we failed to detect it (data not shown). This very short peptide is probably quickly degraded in serum.

In vivo generation of N-terminal midfragment

In plasma assayed within 20 minutes after blood sampling, the N-terminal midfragment represented 76% of the intact osteocalcin level (Table 5). The same proportion was observed in serum taken by a standard procedure. Furthermore, proteinase inhibitors had no effect on the levels of osteocalcin immunoreactive forms measured in plasma within 20 minutes after blood drawing (Table 5).

Degradation of intact osteocalcin in vitro and generation of N-terminal midfragment

We studied the in vitro proteolytic degradation of intact osteocalcin and the generation of the N-terminal midfragment by incubating 10 normal serum samples at room temperature for 2 h with or without proteinase inhibitors. Intact osteocalcin levels were decreased by about 17% ($p <$ 0.001 versus t_0, Table 6), whereas the total level of intact osteocalcin plus N-terminal midfragment measured with

TABLE 4. SERUM LEVELS OF OSTEOCALCIN IMMUNOREACTIVE FORMS IN NORMAL ADULTS AND PATIENTS WITH METABOLIC BONE DISEASE[a]

Subjects	*Intact (nM)*	*N-terminal (nM)*	*Mid (nM)*	*N-terminal mid (nM)*	*Mid C-terminal (nM)*
Normal	2.59 (36.4)[b]	0.99 ± 0.33 (13.9)	1.04 ± 0.15 (14.6)	2.14 ± 0.08 (30.0)	0.36 ± 0.05 (5.1)
Osteoporosis	3.14 (46.2)	0.37 ± 0.11 (5.5)	0.66 ± 0.05 (9.7)	2.22 ± 0.02 (32.7)	0.4 ± 0.07 (5.9)
Chronic renal failure	6.94 (26.3)	2.06 ± 0.22 (7.8)	2.38 ± 0.26 (8.9)	13.37 ± 0.36 (50.6)	1.68 ± 0.05 (6.4)

[a]For each pool (one pool of 15 sera from normal adults, patients with osteoporosis, and patients with chronic renal failure), depletions and assays were performed three times. The results represent mean ± SD of the three runs.
[b]Percentage of the total osteocalcin level.

TABLE 5. N-TERMINAL MID FRAGMENT IN THE CIRCULATION

Sampling procedure	n	Serum osteocalcin (ng/ml)		
		Intact	N-terminal mid	Fragment (% of intact)
Plasma[a]				
Without proteinase inhibitors	6	11.0 ± 3.4	8.0 ± 1.5	76 ± 13
With proteinase inhibitors	6	10.2 ± 2.8	8.2 ± 2.3	80 ± 7
Standard[b]	19	13.7 ± 4.5	10.1 ± 2.9	76 ± 12

[a]Plasma samples were taken on ice, immediately centrifuged, and assayed within 20 minutes.
[b]Standard sampling: blood was taken in tube containing agar-agar, allowed to stand for 60 minutes at 4°C, centrifuged for 10 minutes at 1000 × *g*, and immediately frozen at −80°C.

TABLE 6. IN VITRO PROTEOLYTIC DEGRADATION OF INTACT OSTEOCALCIN AT ROOM TEMPERATURE[a]

	Incubation time		
		2 h Proteinase inhibitors	
	0 h	Without	With
Intact osteocalcin ng/ml	15.1 ± 4.7	12.6 ± 4.8[b]	14.2 ± 4.7
% Loss	—	17[b]	6[c]
Intact + N-terminal mid ng/ml	24.9 ± 7.5	23.8 ± 7.4	25.6 ± 7.7
% Loss	—	4	0

[a]Blood was drawn from 10 normal volunteers under standard sampling procedure (frozen within 1 h 30 minutes), thawed, and assayed immediately or after incubation for 2 h at room temperature with or without proteinase inhibitors.
[b]$p < 0.001$ versus t_0.
[c]$p < 0.005$ versus without proteinase inhibitors (0.2 M ε-aminocaproic acid, 0.01 M benzamidine, 0.001 M PMSF and 0.02 M *N*-ethylmaleinimide).

assay B was not significantly changed (−4%). With proteinase inhibitors, the intact osteocalcin loss was significantly reduced (−6 versus −17% without proteinase inhibitors, $p < 0.005$). Table 7 shows the levels of intact osteocalcin, intact osteocalcin plus N-terminal midfragment, and immunoractive osteocalcin measured with a conventional RIA (RIA osteocalcin) after various incubation times at room temperature. Figure 2 shows the percentage of these various osteocalcin levels before and after 1, 3, 5, and 24 h of incubation at room temperature (Fig. 2A) and at 4°C (Fig. 2B). After 3 h of incubation at room temperature, the total osteocalcin (intact osteocalcin plus N-terminal midfragment) level was not significantly changed (96% of the initial values), whereas about 20% (range 11–31%) of intact osteocalcin and 15% (range 10–22%) of the RIA osteocalcin were lost (Fig. 2A and Table 7). The decrease in intact and RIA osteocalcin levels reached 30% (range 21–37%) and 24% (range 10–30%), respectively, after 5 h and 70 and 55%, respectively, after 24 h of incubation at room temperature (Fig. 2A and Table 7). Maintaining serum at 4°C reduced degradation of the intact and RIA osteocalcin levels in vitro (6 and 3%, respectively, after 3 h), and after 24 at 4°C, the loss was the same as that observed after 3 h of incubation at room temperature (20%; Fig. 2A and B). In contrast, the level of intact osteocalcin plus N-terminal midfragment was more stable at room temperature (−20% after 24 h, Fig. 2A) and at 4°C (−2% after 24 h; Fig. 2B).

Intact osteocalcin and N-terminal midfragment in the supernatant of osteoblastic cells

We measured intact osteocalcin and N-terminal midfragment in the supernatant of MG-63 osteosarcoma cells and human osteoblast-like cells in the presence of 1,25-$(OH)_2D_3$ (10^{-8} M). Figure 3A shows that intact osteocalcin and N-terminal midpeptide were detected in the culture medium of MG-63 cells as early as 6 h, the fragment representing about 28% of the intact osteocalcin level. In addition, the two osteocalcin forms increased in parallel with culture time. After 18 and 24 h the two species were present in the supernatant of human osteoblast-like cells, the N-terminal midfragment representing about 40% of the intact osteocalcin level (Fig. 3B).

TABLE 7. OSTEOCALCIN LEVELS MEASURED WITH THREE DIFFERENT RADIOIMMUNOASSAYS AFTER INCUBATION AT ROOM TEMPERATURE[a]

	Incubation time (h)				
	0	*1*	*3*	*5*	*24*
Intact osteocalcin, ng/ml	12.5 ± 4.0	11.6 ± 3.8	9.9 ± 3.3	8.7 ± 3.0	4.0 ± 1.9
Intact osteocalcin + N-terminal midfragment, ng/ml	21.0 ± 5.6	20.8 ± 5.4	20.3 ± 5.7	18.8 ± 6.1	16.8 ± 5.3
RIA osteocalcin, ng/ml	9.4 ± 2.2	8.7 ± 2.0	8.0 ± 1.9	7.2 ± 2.2	4.3 ± 1.6

[a]Eight normal human serum samples were incubated for 1, 3, 5, and 24 h at room temperature. Osteocalcin was measured with assay A (intact osteocalcin), assay B (intact osteocalcin + N-terminal midfragment), and with a conventional bovine radioimmunoassay (RIA osteocalcin).

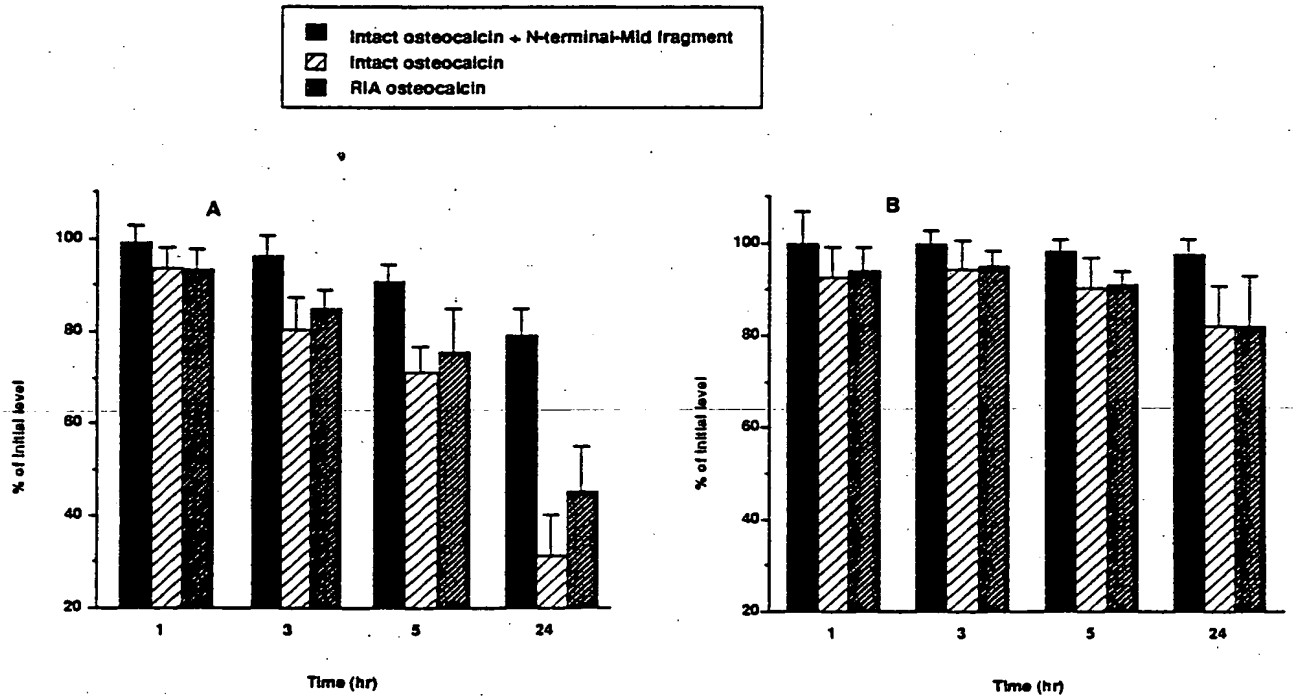

FIG. 2. In vitro proteolytic degradation of intact osteocalcin at room temperature (A) and at 4°C (B). Eight normal human serum samples were incubated for 1, 3, 5, and 24 h at room temperature (A) and at 4°C (B). Osteocalcin was measured with assay A (intact osteocalcin), assay B (intact osteocalcin plus N-terminal midfragment), and a conventional bovine radioimmunoassay (RIA osteocalcin). Data are expressed as the percentage of levels measured without incubation. Bars represent mean ± SD.

Effect of the treatment with pamidroinate of patients with metabolic bone disease on the intact osteocalcin and N-terminal midfragment serum levels

Patients with various metabolic bone diseases were treated for 3 days with pamidronate, and serum intact osteocalcin and N-terminal midfragment were measured before and after treatment (Table 8). After 3 days of treatment the N-terminal midfragment level was not significantly changed for all diseases.

DISCUSSION

Few previous reports have studied the immunoreactive osteocalcin fragments that circulate in addition to the intact molecule. Two studies, using high-performance liquid chromatography fractionation of serum followed by RIA of the fractions have described the presence of multiple immunoreactive osteocalcin fragments of low molecular weight in patients with chronic renal failure[15,17] and Paget's disease,[15] which have been speculated to be released during bone resorption.[17] Using a midmolecule

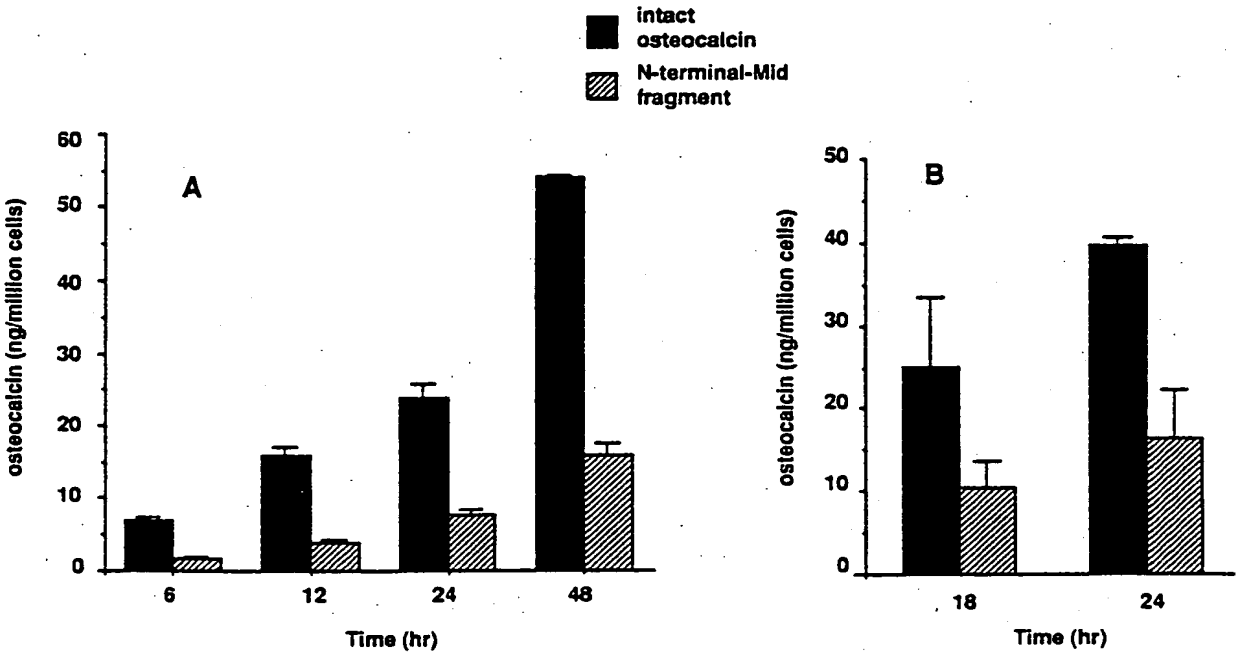

FIG. 3. Time-dependent appearance of intact osteocalcin and N-terminal midfragment in the supernatant of MG-63 cells (A) and human osteoblast-like cells (B). Cells were incubated in FBS-free growth medium supplemented with 0.5% BSA and 10 nM 1,25-$(OH)_2D_3$. Cell media were removed at different times. Intact osteocalcin was measured with assay A and N-terminal midfragment with assay B (intact osteocalcin plus N-terminal midfragment) after removing intact osteocalcin by depletion with MoAb 43–49. Points represent mean ± SD of three separate experiments.

TABLE 8. SHORT-TERM EFFECT OF IV PAMIDRONATE TREATMENT ON THE CIRCULATING LEVELS OF INTACT OSTEOCALCIN AND N-TERMINAL MIDFRAGMENT IN PATIENTS WITH METABOLIC BONE DISEASE[a]

		Intact osteocalcin (ng/ml)		*N-terminal-midfragment (ng/ml)*		
Subjects	n	*Before treatment*	*After treatment*	*Before treatment*	*After treatment*	p[b]
Paget's disease	7	17.5 ± 6.3	20.3 ± 6.6	16.2 ± 6.7	18.8 ± 7.4	NS
Reflex sympathetic dystrophy	8	13.4 ± 5.7	11.0 ± 4.5	18.6 ± 9.0	16.8 ± 5.6	NS
Fibrous dysplasia	4	25.5 ± 13.2	27.4 ± 16.0	37.1 ± 16.3	36.7 ± 15.9	NS
Osteoporosis	4	9.6 ± 1.2	9.8 ± 4.2	11.2 ± 1.7	11.4 ± 4.8	NS

[a]Patients with metabolic bone disease were treated by 60 mg/day of the bisphosphonate pamidronate for 3 days. Blood was taken before and after treatment on day 4, and intact osteocalcin was measured with radioimmunoassay A (intact osteocalcin) and N-terminal midfragment with radioimmunoassay B (intact osteocalcin plus N-terminal midfragment) after depletion in intact osteocalcin by incubation with monoclonal antibody 43–49. Mean ± SD.

[b]After treatment versus before treatment for intact osteocalcin and N-terminal midfragment.

osteocalcin RIA, Taylor et al.[15] described immunoreactive fragments in normal and pathologic serum and urine samples. In addition, osteocalcin-related peptides found in the serum of patients with chronic renal failure correspond to some of those found in normal serum and urine, indicating that some fragments are generated in the circulation before renal clearance.[15] Recently, Tracy et al.,[19] using a MoAb-based RIA, also reported the presence of low-molecular-weight osteocalcin fragments in normal serum that were not related to bone resorption. None of these studies provided information about the structure and origin of these fragments, and our work is the first comprehensive characterization of the various immunoreactive forms of circulating human osteocalcin. Using three MoAb recognizing regions that span the linear sequence of osteocalcin, specifically 5–13 (N-terminal), 25–37 (mid), and 43–49 (C-terminal) in several RIA, we detected four fragments, that is, N-terminal, mid, N-terminal mid, and mid C-terminal, in the serum of normal adults and patients with metabolic bone disease. We cannot exclude the presence of other

fragments resulting from cleavages at sites other than peptide bonds between amino acids 19–20 and 43–44, in addition to those we detected, although these two bonds are the most likely sites of cleavage from the sequence.[21] The C-terminal fragment was indetectable in serum with a specific RIA for the 43–49 synthetic peptide, suggesting that this very short fragment is rapidly degraded in serum and excreted from the circulation. N-terminal, mid, and mid C-terminal fragments, resulting probably from the cleavage of the 19–20 peptide bond, were detected in minute amounts compared to that generated from the osteocalcin breakdown in the 43–49 position, that is, the N-terminal midfragment. It is likely that the 19–20 arginine-arginine bond is less sensitive to proteolytic cleavage than that found in the 43–49 position. According to the model proposed by Hauschka et al.[28] the former is engaged in a gla helix, whereas the latter is situated in the COOH-terminal β sheet, which is probably more accessible to proteolytic degradation. The N-terminal midfragment is the more prominent: it was detected in large amounts in normal and pathological serum, and there is some evidence that its level is not overestimated. This peptide was measured with assay B, using intact human osteocalcin as a standard, after depletion of serum in intact osteocalcin with MoAb 43–49. We previously showed[18] that for assay B, the recovery of intact human osteocalcin spiked in normal human serum (containing a large amount of N-terminal midfragment) was very good (ranging from 96 to 103%). This suggests that this assay recognized intact human osteocalcin used as a standard and N-terminal midfragment with the same affinity. Thus, the levels of N-terminal midfragment were accurate and may be compared to those of intact osteocalcin.

To investigate whether these immunoreactive osteocalcin forms are generated in vivo, we measured the most abundant N-terminal midfragment in plasma taken under conditions avoiding any in vitro proteolytic degradation. Assaying plasma within 20 minutes after sampling in the presence of proteinase inhibitors, this fragment still represented 76% of the intact osteocalcin level, indicating that it circulates in vivo. In addition this fragment was detected in the supernatant of MG-63 cells and human osteoblast-like cells. The N-terminal midfragment may be secreted by osteoblastic cells after intracellular processing of the intact protein since there is no evidence for sites leading to mRNA other than that for intact molecule in the human osteocalcin gene[29] and/or generated from proteolytic degradation of the 49-residue mature protein in the culture media. Several previous studies have shown that no osteocalcin-related peptides are detected after controlled extraction of bovine or human bone,[6,30,31] whereas several fragments have been described in normal and pathologic serum[15,17,19] suggesting that osteocalcin is metabolized in the circulation after secretion of the intact protein by osteoblasts in the bone. Levels of N-terminal midfragment were not altered after treatment of patients with various metabolic disease (Paget's disease, osteoporosis, reflex sympathetic dystrophy, and fibrous dysplasia) with the bisphosphonate pamidronate, a potent and specific inhibitor of bone resorption, suggesting that this large peptide is not released into the circulation during osteoclastic degradation of bone matrix.

After incubating normal serum at room temperature for various times, we observed a continuous decrease in osteocalcin levels measured with the IRMA for the intact molecular and with a conventional bovine RIA, whereas the total intact osteocalcin plus N-terminal midfragment level was not significantly altered until 24 h of incubation. This intact osteocalcin degradation was partially inhibited by proteinase inhibitors and by keeping serum at 4°C after sampling. This suggests that intact osteocalcin is rapidly degraded in vitro at room temperature by proteolytic cleavage, generating mainly the large N-terminal midfragment. In addition, the RIA osteocalcin level was about two- to threefold lower than intact osteocalcin plus N-terminal midfragment serum level and decreased rapidly after incubation of serum at room temperature, suggesting that conventional bovine RIA recognize preferentially the intact molecule and poorly the large N-terminal midfragment. These results are in agreement with our previous study[18] and with the results of Price and Nishimoto[6] who showed that the 1–40 peptide derived from a carboxypeptidase Y digestion was not detected by another bovine RIA and that the COOH-terminal region was required for osteocalcin recognition by that polyclonal antiserum. This in vitro proteolytic degradation of osteocalcin due to inadequate sampling procedures probably explains some discordant previous results obtained with conventional RIA.

In all pooled serum samples, the N-terminal midfragment was the most abundant, representing about 30% of the total osteocalcin level in normal adults and patients with osteoporosis and 50% in patients with chronic renal failure. In patients with postmenopausal osteoporosis, the concentration of the various osteocalcin-immunoreactive forms were the same as those observed in controls, whereas they were increased in patients with Paget's disease and chronic renal failure because of an increase in bone turnover.[7,9,16,18,20] In patients with chronic renal failure, the N-terminal midfragment was accumulated over the others (sixfold the normal concentration), probably because the renal clearance of this large fragment is altered in this disease.

In conclusion, we characterized multiple serum osteocalcin immunoreactive fragments using specific RIA and depletions. Among the immunoreactive forms, the N-terminal midfragment was the most abundant. These fragments circulated in the serum of normal adults and patients with metabolic bone disease probably generated from proteolytic degradation of the intact molecule in the circulation. In addition, they can also be obtained in vitro from the degradation of intact osteocalcin, which appears to be a very unstable peptide. To obtain accurate and reliable osteocalcin levels measured either with an IRMA specific for intact osteocalcin or with conventional RIA, it is necessary to control the sampling conditions, including maintaining blood samples at 4°C immediately after sampling and freezing serum or plasma within 1 h.

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REFERENCES

1. Hauschka PV, Lian JB, Gallop PM 1975 Direct identification of the calcium-binding amino acid gamma-carboxyglutamic in mineralized tissue. Proc Natl Acad Sci USA 72: 3925–3929.
2. Price PA, Otsuka D, Poser JW, Kristaposis J, Raman N 1976 Characterization of a gamma-carboxyglutamic containing protein from bone. Proc Natl Acad Sci USA 73:1447–1451.
3. Lian JB, Friedman PA 1978 The vitamin K-dependent synthesis of γ carboxyglutamic acid by bone microsomes. J Biol Chem 253:6623–6626.
4. Gallop PM, Lian JB, Hauschka PV 1980 Carboxylated calcium-binidng protein and vitamin K. N Engl J Med 302: 1460–1466.
5. Lajeunesse D, Frondoza C, Schoffield B, Sacktor B 1990 Osteocalcin secretion by the human osteosarcoma cell line. J Bone Miner Res 5:915–922.
6. Price PA, Nishimoto SK 1980 Radioimmunoassay for the vitamin-K dependent protein of bone and its discovery in plasma. Proc Natl Acad Sci USA 77:2234–2238.
7. Price PA, Parthermore, JG, Deftos LJ 1980 New biochemical marker for bone metabolism: Measurement by radioimmunoassay for gla protein in the plasma of normal subjects and patients with bone disease. J Clin Invest 66:878–883.
8. Delmas PD, Wahner HW, Mann K, Riggs BL 1983 Assessment of bone turnover in postmenopausal osteoporosis of serum bone gla protein. J Lab Clin Mes 102:470–476.
9. Gundberg CM, Lian JB, Gallop PM, Steinberg JJ 1983 Urinary γ carboxyglutamic acid serum osteocalcin as bone markers: Studies in osteoporosis and Paget's disease. J Clin Endocrinol Metab 57:1221–1225.
10. Deftos LJ, Parthermore JG, Price PA 1982 Changes in plasma bone gla protein during treatment of bone disease. Calcif Tissue Int 34:121–124.
11. Delmas PD, Wilson DM, Mann KG, Riggs BL 1983 Effects of renal function on plasma levels of bone gla protein. J Clin Endocrinol Metab 57:1028–1030.
12. Brown JP, Delmas PD, Malaval L, Edouard C, Chapuy MC, Meunier PJ 1984 Serum bone gla protein. A specific marker for bone turnover in postmenopausal osteoporosis. Lancet 1: 1091–1093.
13. Price PA, Williamson MK, Lothringer JW 1981 Origin of a vitamin K-dependent bone protein found in plasma and its clearance by kidney and bone. J Biol Chem 256:12760–12766.
14. Farrugia W, Melik RA 1986 Metabolism of osteocalcin. Calcif Tissue Int 39:234–238.
15. Taylor AK, Linkart S, Mohan S, Chrinstenson RA, Singer FR, Baylink D 1990 Multiple osteocalcin fragments in human urine and serum as detected by a midmolecule osteocalcin radioimmunoassay. J Clin Endocrinol Metab 70:467–472.
16. Hosoda K, Eguchi H, Nakamoto T, Kubota T, Honda H, Jindai S, Hasegawa R, Kiyoki T, Shiraki M 1992 Sandwich immunoassay for intact human osteocalcin. Clin Chem 38: 2233–2238.
17. Gundberg CM, Weinstein RS 1986 Multiple immunoreactive forms of osteocalcin in uremic serum. J Clin Invest 77:1762–1767.
18. Garnero P, Grimaux M, Demiaux B, Préaudat C, Seguin P, Delmas PD 1992 Measurement of serum osteocalcin with a human-specific two-site radioimmunoassay. J Bone Miner Res 7:1384–1398.
19. Tracy RP, Andrianovo A, Riggs BL, Mann KG 1990 Comparison of monoclonal and polyclonal antibody-based for osteocalcin: A study of sources of variation in assay results. J Bone Miner Res 5:451–461.
20. Delmas PD, Christiansen C, Mann KJ, Price PA 1990 Bone gla protein (osteocalcin) assay standardization report. J Bone Miner Res 5:5–11.
21. Price PA, Poser JW, Raman N 1976 Primary structure of the γ-carboxyglutamic acid-containing protein from bovine bone. Proc Natl Acad Sci USA 73:3374–3375.
22. Greenwood FG, Hunter WN, Glover JS 1963 The preparation of ^{131}I-labeled human growth hormone. Biochem J 89: 114–120.
23. Tarallo P, Henny B, Fournier B, Siest G 1990 Plasma osteocalcin: Biological variations and reference limits. Scand J Clin Lab Invest 50:649–655.
24. Pietschmann P, Woloszczuk W, Panzer S, Kyrle P, Smolen J 1988 Decreased serum osteocalcin levels in phenprocoumon-treated patients. J Clin Endocrinol Metab 66:1071–1074.
25. Sebert JI, Ruiz JC, Fournier A, Fardellone P, Guéris J, Marié A, Morinière P, Codet MP, Renaud H 1987 Plasma bone gla-protein: Assessment of its clinical value as an index of bone formation in hemodialysed patients. Bone Miner 2: 21–27.
26. Bergmann, Valsamis, J, Van Perborgh J, De Schepper J, Van Vielt G 1990 Comparative study of the changes in insulin-like growth factor-I, procollagen-III N-terminal extension peptide, bone gla-protein, and mineral content in children with Turner's syndrome treated with recombinant growth hormone. J Clin Endocrinol Metab 71:1461–1467.
27. Chavassieux PM, Chenu C, Valentin-Opran A, Merle B, Delmas PD, Hartmann DJ, Saez S, Meunier PJ 1990 Influence of experimental conditions on osteoblast activity in human primary bone cell cultures. J Bone Miner Res 5:337–343.
28. Hauschka PV, Lian JB, Cole DEC, Gundberg CM 1989 Osteocalin and matrix gla protein: Vitamin K-dependent proteins in bone. Physiological Reviews 69:990–1097.
29. Celeste AJ, Rosen V, Buecker JL, Kriz R, Wang EA, Wosney JM 1986 Isolation of the human gene for bone gla protein utilizing mouse and rat cDNA clones. EMBO J 5:1885–1890.
30. Power MJ, Gosling JG, Fottrell PF 1989 Radioimmunoassay of osteocalcin with polyclonal and monoclonal antibodies. Clin Chem 35:1408–1415.
31. Gundberg CM, Hauschka PV, Lian JB, Gallop PM 1984 Osteocalcin: Isolation, characterization and detection. Methods Enzymol 107:516–544.

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